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The International Union Against Cancer

A REPORT BY THE U. S. A. NATIONAL COMMITTEE ON THE INTERNATIONAL UNION AGAINST CANCER, *National Research Council,*[1] *Washington, D. C.*

The first attempt to create a world-wide cancer organization was made before World War I under the auspices of "The International Association for Cancer Research." This organization was officially dissolved when Professor J. Fibiger of Copenhagen, Denmark, resigned as president of the 4th International Congress, which was to be held in Copenhagen about the time the first world war began. This association had been very active. Three international congresses were organized (1907, 1910, and 1913), and an active international campaign was carried out to educate the public in matters dealing with cancer.

Following World War I, the first sign of the revival of international activity in the field of cancer study was the organization of a conference at Strasbourg, in 1923, on the occasion of the centennial anniversary of the birth of Pasteur.

Three years later, in 1926, Doctor F. L. Soper, then Secretary, and Doctor Howard C. Taylor, President of the American Society for the Control of Cancer, organized an international symposium at Lake Mohonk, New York, U. S. A., which was attended by representatives of eight nations. This symposium, a model of its kind, was a great success.

In 1928, the British Empire Cancer Campaign organized another international conference in London, attended by representatives of sixteen nations. Discussions were held regarding dissemination of information about cancer to the public, and for the first time the problem of the geographic and racial distribution of cancer was discussed.

[1] This report is also being published in the journals *Cancer Research* (September 1952) and *Cancer* (November 1952).

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A successful attempt to establish a permanent international group after World War I (which had been suggested at the meeting in America in 1926) was made in Madrid, Spain, in 1933 by the French Minister of Health, Justin Godart, and Doctor Jacques Banadaline. This first meeting of the newly formed International Congress Against Cancer was concerned with the scientific aspects of cancer as well as the aspects of its control. As a result, the International Union Against Cancer (Union Internationale Contre le Cancer, UICC) was created in 1933 with Godart as President, Paris as the headquarters, and the bylaws registered under French law. The second Congress organized by the Union was held in Brussels, Belgium, in 1936 and the third in Atlantic City, New Jersey, U. S. A. in 1939.

The accomplishments of this international group up to this time have been the organization of Cancer Congresses; the establishment of an international journal, *Acta Unio Internationalis Contra Cancrum*, edited by Professor J. Maisin, Voer des Capucins, 61, Louvain, Belgium, to publish the proceedings of the Congresses; the encouragement of studies in geographic pathology; the international dissemination among scientists and the public of information dealing with cancer; and some recommendations for a central abstracting center for cancer literature, for the preparation of standard cancer nomenclature, and for atlases of tumor pathology. Recently, in the United States the American Cancer Society has published its *Manual of Tumor Nomenclature and Coding*, which is now in the Statistical Committee of WHO for international adoption. The *Atlases of Tumor Pathology* are currently in preparation under the auspices of the Subcommittee on Oncology of the National Research Council.

During World War II, all activities of the International Union Against Cancer ceased. Following the war, the Union accepted the invitation of the American Association for Cancer Research to hold the 4th International Cancer Research Congress in Saint Louis, U. S. A., in 1947 and Doctor E. V. Cowdry was selected as president. The 5th Congress was held in Paris, France, in 1950 and the 6th is scheduled to be held in São Paulo, Brazil, in January 1954. (*This date may be changed.*)

The International Union Against Cancer has been represented in the World Health Organization (WHO), and in the United Nations Educational, Scientific and Cultural Organizations (UNESCO) through the Council for International Organization of Medical Sciences (CIOMS).

The International Cancer Research Commission

At the Saint Louis meeting of the Union in 1947, the International Cancer Research Commission (ICRC) was created as an outgrowth of the Scientific Committee of the Union. This move reflects the expanding activity in the field of cancer research throughout the world. The Commission has greater autonomy within the Union than had the Scientific Committee. The principles of the Commission as originally stated embrace all efforts to advance our knowledge of cancer by clinical and experimental means. The Commission as an advisory body will help to plan and promote cancer research, and it is largely responsible for developing

the field of geographic pathology, for standardizing the nomenclature of cancer, and for indexing of cancer literature, all at the international level. The first president of the Commission was Doctor I. Milan of Mexico and the current president is Professor V. R. Khanolkar of Bombay, India. The last meeting of the Commission was held in Lisbon, Portugal, on 15–20 December 1951. The Executive Committee of the International Union Against Cancer met at the same time, also in Lisbon.

Summary of the Lisbon Meetings December 1951

Because some important decisions were made and some clearly defined resolutions were offered at the meeting of the Executive Committee of the Union and the meeting of the Scientific Commission (International Cancer Research Commission) at Lisbon, Portugal, in December of 1951, a summary of this meeting is presented.

In the Executive Committee of the International Union Against Cancer it was proposed to create within the structure of the Union an international committee to concern itself with problems relating to the control of cancer. This was voted on and passed unanimously. The Executive Committee appointed the following members to draft the aims, objectives, and duties of such a committee:

Professor Xenophon Chahovitch, Belgrade, Yugoslavia
Professor J. H. Maisin, Institut du Cancer, Louvain, Belgium
Professor J. L. Nicod, Université de Lausanne, Lausanne, Switzerland
Professor Nicholas Puente-Duany, de l'Institut du Radium, Cuba
Doctor Charles S. Cameron, American Cancer Society, New York, New York, U. S. A.

The Executive Committee realized that to ensure a more effective functioning of the UICC, greater financial stability was desirable. It was voted that participating countries and organizations should be approached and a plea made for an increase in the contributions to the Union.

At the Lisbon meeting, the ICRC elected a committee on Geographic Pathology and one on Nomenclature, approved a bibliographic center for indexing and documentation of cancer literature, and adopted the following resolutions primarily concerned with the membership of the Commission and future meeting dates:

1) That National Representatives to the Commission should be nominated by their governments with the advice and support of either cancer research organizations or medical faculties.

2) That the program of the meetings will be drawn up by the Executive Committee of the International Cancer Research Commission assisted by the Union and a program committee.

3) That one member of the Executive Committee shall be appointed as Secretary of the ICRC for the period of his tenure on the Executive Committee.

4) That there shall be created a new category of membership, Associate Member, to be nominated upon the recommendation of the chairmen of the committees; these members are invited to take part in the work of the committees and in the meetings of the Commission, but shall not be entitled to vote.

5) That the International Congress on Cancer at São Paulo, Brazil, shall commence on 3 January 1954. (*This date may be changed.*)

6) That meetings of the Executive Committee of the Union and of the ICRC shall immediately precede the meeting of the International Congress on Cancer at São Paulo.

7) That the next meeting of the ICRC shall be in Bombay, India, in December 1952 and that in the future only one meeting will be held during the intervening period between two International Congresses.

8) That the annual subsidy granted by the Union to the Commission shall be raised from its present level of $1,200 to $2,400.

The headquarters of the Committee on Geographic Pathology was established at the National Cancer Institute, Bethesda, Maryland, U. S. A., and the following members were elected to the Committee:

Professor Johannes Clemmesen, *Associate Secretary*
The Danish Cancer Registry, Copenhagen, Denmark

Doctor Harold L. Stewart, *Executive Secretary*
Chief, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland, U. S. A.

Professor V. R. Khanolkar, *Ex Officio*
Executive Officer, Tata Memorial Hospital, Bombay, India

Doctor Pierre Denoix
National Institute of Hygiene, Paris, France

Professor Joseph Gillman
Medical School Hospital, Johannesburg, South Africa

Professor J. H. Maisin
Institut du Cancer, Louvain, Belgium

The following members were elected to the Committee on Nomenclature:

Doctor Isabella H. Perry, *Chairman*
University of California Medical School, San Francisco, California, U. S. A.

Doctor Pierre Denoix, *Secretary*
National Institute of Hygiene, Paris, France

Professor E. Berven
Radiumhemmet, Stockholm, Sweden

Professor Herwig Hamperl
Direktor des Pathologischen Institutes der Universität Marburg, Marburg/Lann, Germany

Doctor Cecil Jackson
Medical School Hospital, Johannesburg, South Africa

Professor Mathias Roxo-Nobre
Clinico da Clinica da Associacão Paulista de comb. ao Cancer, São Paulo, Brazil

Doctor C. Chester Stock
Sloan-Kettering Institute for Cancer Research, 410 East 68th Street, New York, New York, U. S. A.

It was proposed: that a World Cancer Day be observed and that all necessary measures be taken to ensure its success; that the issuance of an international stamp for the campaign against cancer be approved; and that the issuance of a special stamp for future International Congresses in the countries in which they will be held be approved.

The two committees of the ICRC mentioned above reported to the Commission at the Lisbon meetings, and some of the points discussed and agreed upon are of sufficiently broad interest to warrant presentation here.

Committee on Geographic Pathology

The Committee on Geographic Pathology has defined its field of interest as comprising knowledge concerning variations in the distribution and behavior of cancer among various ethnologic groups in different localities in relation to local factors.

The Committee agreed that its primary functions would be:

1) To serve as a point of contact and communication among investigators in its field of interest throughout the world.

2) To invite the co-operation of cancer investigators to serve as correspondents in order that centers of information in the various countries of the world might be established.

3) To encourage and facilitate co-operation on specific scientific problems by assisting in the procurement of grants-in-aid for research.

4) To call small groups of experts together for symposia concerned with particular problems in the geographic pathology of cancer.

5) To promote the publication of information on the endemiology of cancer in journals devoted to cancer research.

Committee on Tumor Nomenclature

Dr. Isabella Perry, Chairman of the Committee on Tumor Nomenclature, had forwarded to members of the Committee a provisional draft of the *Manual of Tumor Nomenclature and Coding* published by the American Cancer Society. The members had voted to approve the manual and the Committee recommended that, for the present, work on a code for the clinical stages of carcinoma be confined to accessible sites. It was agreed that the criteria for determining the clinical stages of carcinoma for accessible sites would be the physical examination supplemented by such instrumentation as is in general use by the practitioner

and not such as to require consultation with a specialist. The following general code for the clinical stages of accessible carcinoma was proposed:

Primary Site

0... No tumor.

I... Tumor (relatively large size) infiltrating the primary organ site.

II... Tumor of relatively larger size, infiltrating the primary organ site and not extending beyond the adjacent soft tissues.

III.. Tumor widely infiltrating the primary organ site and extending to neighboring organs.

IV.. Tumor extensively involving adjacent soft tissues or organs.

Metastases

0... None.

I... Regional lymph nodes slightly enlarged, discrete and movable.

II... Regional lymph nodes considerably enlarged, discrete and movable.

III.. Regional lymph nodes enlarged, and fixed either to one another or to adjacent structures.

A... Clinical stages confirmed microscopically.

This Committee felt that there is considerable evidence of a need for a clearer and more uniform nomenclature and recording system for tumors. It was pointed out that WHO has established a "clearing center for problems arising in the application of the *International Statistical Classification*" and a "focal unit" for maintaining relationships with national committees or other organizations of national scope. The Committee recommended approval of the "clearing center" and "focal unit" and entered a plea to be sent to the Chairman of the WHO *Subcommittee on the Registration of Cases of Cancer as Well as Their Statistical Presentation* that the "center" work on these problems.

United States of America National Committee on the International Union Against Cancer

As a result of the increased activity of the International Union Against Cancer since World War II, there has been a growing feeling on the part of individuals and organizations in the United States with an interest in cancer that some group should be formed to organize, coordinate, and strengthen the participation of the United States in the International Union. In April 1951, Doctor William U. Gardner was selected for the second time to serve as American Representative to the International Research Commission of the Union by the American Association for Cancer Research. Doctor Gardner requested and received the authorization of this group to discuss with other organizations interested in cancer the suggestion that representation of the United States in the Union be form-

alized through the establishment of a perpetuating organization affiliated with the National Research Council of the United States.

This suggestion was agreeable to individuals and agencies interested in cancer and in the Union. As a result, a meeting of representatives of the American Association for Cancer Research, the American Cancer Society, the International Union Against Cancer, the National Cancer Institute, and the National Research Council was called on 21 October 1951 to consider ways and means of establishing a group to facilitate representation and participation of the United States in the Union. At this meeting the National Research Council was requested to establish the United States of America National Committee on the International Union Against Cancer consisting of at least five members selected from nine nominations, three each of which would be submitted by the American Association for Cancer Research, the American Cancer Society, and the National Cancer Institute. Each member would serve for a period of five years, except that the original members would be appointed for staggered terms so that thereafter one new member would be appointed each year from nominations submitted by the three agencies mentioned above.

It was agreed that the general functions of this Committee would be:

1) To select and instruct representatives to meetings of the Union and Commission and to receive reports from these representatives.

2) To devise means for the support of the functions of the Committee, its delegates and representatives in relation to the general and specific functions.

3) To establish an office or facilities for the transmission of information relating to cancer to the Union or Commission or its members in the several countries and to receive similar material for appropriate distribution in the United States of America.

4) To approve all groups from the United States that may apply for membership in the Union and otherwise to encourage membership in the Union so as to foster its activities.

Subsequently the National Research Council agreed to establish the U. S. A. National Committee on the International Union Against Cancer in the Division of International Relations and in close liaison with the Division of Medical Sciences. The following members have been appointed:

Doctor William U. Gardner, *Chairman*, Yale University
Doctor Charles S. Cameron, American Cancer Society
Doctor E. V. Cowdry, Washington University
Doctor John R. Heller, National Cancer Institute
Doctor Paul E. Steiner, University of Chicago

At its first meeting on 3 March 1952, the Committee accepted and approved the report by Doctor Harold L. Stewart, National Cancer Institute, who was the official representative of the United States at meet-

ings of the Union and the Commission in Lisbon, Portugal on 15–20 December 1951. The Committee also drafted a Constitution and discussed the selection and instruction of delegates to meetings of the Union and ways of obtaining financial support for the Union. The Committee has had two subsequent meetings and at its latest on 16 June 1952 plans were made for the participation of six delegates from the United States in a "Symposium on the Endemiology of Lung Cancer" held in Louvain, Belgium, on 21–24 July 1952. The Committee also appointed Doctor Harold L. Stewart of the National Cancer Institute to represent the United States at the next meeting of the International Cancer Research Commission to be held in Bombay, India, in December 1952 or in January 1953.

There are three categories of membership within the Union—namely, countries, private organizations, and individuals—requiring membership fees of $250.00, $100.00, and $10.00 respectively. It has been agreed with the Union that membership in the Union from the United States shall be approved by the National Committee. The members of the Committee are anxious to do everything possible to increase interest in and support of the International Union Against Cancer. Questions, suggestions, or requests for further information should be directed to the National Committee on the International Union Against Cancer, National Research Council, 2101 Constitution Avenue, Washington 25, D. C., U. S. A.

REFERENCES

(*1*) Minutes of the meeting of the Executive Committee of the International Union Against Cancer, held at Lisbon, Portugal, 15 December 1951.

(*2*) Report of the Secrétaire Général, Prof. J. Maisin, to the General Assembly of the Union Internationale Contre le Cancer, Paris, France, 18 July 1949.

Experimental Studies in Metal Cancerigenesis. II. Experimental Uranium Cancers in Rats [1]

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In view of the rapidly increasing mining and milling of uranium ores carried on in this country and abroad, it appeared to be important to investigate whether or not cancers might develop from a tissue into which metallic uranium was placed in the form of a depot, producing, thereby, a high local concentration of uranium over a long period of time. Moreover, a study of this problem appeared to be pertinent since, according to Dounce (*1*), "Nothing is known as to whether individual cells here and there might be affected by radiation as the result of the accidental accumulation of very high concentrations of uranium in their vicinity. The latter possibility could become important in its relationship to carcinogenesis, but there is thus far no evidence that uranium compounds are carcinogenic."

EXPERIMENTAL PROCEDURE

Powdered metallic uranium was procured. In tests subsequently performed, it was shown that the uranium used was a pure alpha-ray emitter. In the experiments to be described, the powdered uranium was incorporated into lanolin. The suspension contained 25 percent uranium by volume and 60 percent by weight. There were approximately 50 mgm. of uranium in 0.05 cc. of the suspension.

EXPERIMENT I. INTRAFEMORAL INJECTION OF METALLIC URANIUM

Thirty-three albino rats, Osborne-Mendel strain, were used. Twenty-five of them were males and 8 were females. The males were 4 months old and the females were 6 months old at the start of the experiment. For the introduction of the uranium suspension into the right femur, a small hole was drilled into the lower end of the femur with an electric drill having a flexible shaft and a No. 1 dental bit. Roughly 0.05 cc. of the suspension was injected into the femoral cavity under ether anesthesia. This uranium

[1] Received for publication January 18, 1952.
[2] National Institutes of Health, Public Health Service, Federal Security Agency.

paste was made semiliquid by keeping it, as well as the 1 cc. syringe, in warm water until the injection was performed. There was almost always a certain amount of back leakage of the uranium paste into the muscle and periosteal tissues. The incision of the skin was closed by one or two silk sutures. These operations were well tolerated. There were no acute postoperative deaths.

The experiment was started in August 1949 and the last 5 surviving rats were sacrificed in August 1951. Blood examinations were made during the first 9 months at 2-month intervals. There were no remarkable changes in the number of leukocytes and erythrocytes, or in the amount of hemoglobin. While the differential counts of the white cells did not reveal any appreciable or consistent variations in most of the various cell types, a mild increase in the number of eosinophilic cells seemed to occur toward the end of the observation period. There were 2.9 percent eosinophilic leukocytes at the beginning of the experiment. This percentage rose to 4.6 at 9 months after the intrafemoral injection of uranium. With advancing age the rats displayed a moderate general loss of hair, which sometimes was most marked in the region of the right thigh.

Three of the 33 rats died within the first 6 months, 9 during the following 6-month period, and 21 died or were sacrificed during the last 12 months of a total observation period of 24 months.

Necropsy observations.—Necropsies were performed on all rats. With the exception of swollen kidneys with diffuse small, yellow cortical spotting, and pulmonary congestion, there were no abnormal changes in the 3 rats that died during the first 6-month postinjection period. Among the 9 rats of the second group, that died during the 7- to 12-month period, 2 had tumors of the right thigh that surrounded the femur and measured $5 \times 5 \times 5$ cm. and $3.5 \times 2.5 \times 2.5$ cm., respectively. The latter tumor was firmly attached to the proximal end of the femur as well as to the pelvic bone, and showed upon X-ray examination a dense shadow with a fine trabecular structure (fig. 1). Both tumors and their metastatic growths (in the inguinal and retroperitoneal lymph nodes and in the lung, respectively) revealed a white, homogeneous, baconlike cut surface and were of firm consistency. Two additional rats were found to have abscesses filled with a creamy, yellowish liquid matter and measuring about 2 cm. in diameter in the muscle tissue of the right thigh. A well encapsulated, medium soft, nodular tumor was found in the subcutaneous tissue of the left thoracic wall of a fifth rat of this group. Small black deposits were frequently found within the muscle tissue of the right thigh.

Of the 21 rats that survived for 13 to 24 months, 9 developed tumors at the site of injection. These varied in size from small, round, firm nodes with white and black parts, measuring less than 1 cm. in diameter, to tumor masses measuring $6.5 \times 4.5 \times 4$ cm. Six of the tumors measured more than 1.5 cm. in diameter. The tumors, as a rule, were separated by a fibrous capsule from the skin, but were firmly adherent to the femur. In one instance, the firm white homogeneous tumor mass infiltrated diffusely the surrounding muscle tissue and was not attached to the femur.

In 3 of the tumors, cheesy black matter was found embedded in the tumor tissue. The centers of the larger nodes were reddish, discolored, soft and, in part, liquefied. Multiple, white, medullary, well circumscribed nodes measuring from 0.5 cm. to 3 cm. in diameter were present in the lungs of 3 tumor-bearing rats, one of which also had enlarged, white, medullary mediastinal lymph nodes. A rather soft, encapsulated tumor, measuring $4 \times 5 \times 3.5$ cm. and having a hemorrhagic central portion, was found at the mesenteric radix of one rat. One of the rats with tumors in the thigh had, moreover, an oval-shaped, soft, well encapsulated nodular neoplasm measuring about 2.5 cm. in diameter, situated in the subcutaneous tissue of the right inguinal region and showing a fibrillar texture on the cut surface. Two nodes of similar size and appearance were present in the right inguinal and right axillary regions of a non-tumor-bearing rat.

Many of the treated rats showed extensive grayish pink to dark red pneumonic indurations of the lungs. A fine granular cortex and small cortical cysts were noted in the kidneys of 3 rats, and 3 other rats had small, soft, collapsed testes.

Histology.—In the rats that died during the first 6 months of the experimental period, the muscle tissue of the thigh showed extensive areas of muscular necrosis around black amorphous deposits. There was a moderate accumulation of leukocytes and fibroblasts in the periphery of the necrotic areas. Where clumps of black, granular matter identical with the uranium injected were located within the subcutaneous tissue, an inflammatory granulation tissue and a hyperplastic and hyperkeratotic epidermis were noted.

The endosteal and periosteal tissue near the uranium deposits showed an active fibroblastic proliferation and the formation of new osteoid trabecular bone. In one rat these productive periostitic reactions were appreciable and displayed a considerable degree of cellular and nuclear irregularity.

Of the treated rats that died subsequently, two had large cavities lined by a chronically inflamed and highly vascularized granulation tissue enclosing necrotic matter. The epidermis above these abscesses was hyperplastic. Uranium deposits in the muscle tissue of these and other rats were encysted and surrounded by hyaline tissue, when the uranium occurred in rather large, solid clumps. In other deposits the uranium was dispersed as a fine powder within large phagocytic cells that formed granulomatous masses within the muscle tissue (figs. 2 and 3). The arterioles and small arteries within or adjacent to such foci had thickened and hyaline walls. Uranium deposits in the marrow cavity of the femur were surrounded by necrotic bone and hyalinized and fibrotic marrow (fig. 4). The periosteum and sometimes, also, the endosteum showed an appreciable fibroblastic thickening and proliferation of trabecular bone.

In the tumors involving the site of injection, *i. e.* the right femur, the surrounding muscle tissue and adjacent structures (pelvic bone and spine) as well as the metastatic growths in lung and lymph nodes were composed, with two exceptions, of irregularly arranged strands, bundles, or masses

of slender or plump oval or spindle-shaped cells, which involved, in most instances, the periosteal tissue and infiltrated diffusely into the surrounding muscle tissue (figs. 5 and 6). The nuclei were hyperchromatic and mitoses were present in moderate number. They were, thus, spindle-cell sarcomas. In some areas there was a considerable amount of intercellular fibrillar tissue present. In two tumors a trabecular bone formed the matrix in which sarcoma cells were embedded (fig. 7). The pulmonary metastases of one of these tumors reproduced the bone-forming structures of the primary growth, while the abdominal mass associated with the second bone-producing sarcoma consisted of slender spindle cells sometimes arranged in palisade formation and embedded in places in a hyaline stroma (fig. 8). Uranium deposits were frequently found within the sarcomatous tissue.

The subcutaneous, nodular, encapsulated tumors located in the mammary region of female rats were composed of glandular nodules surrounded by fibrillar connective tissue and, thus, were typical adenofibromas of the breast, which not infrequently occur spontaneously in rats of various strains.

Apart from nonspecific inflammatory, congestive, and degenerative reactions seen especially often in the lung and, to a lesser extent, in the liver, pathologic changes causally related to the uranium injected were found in the kidneys of the 3 rats that died within a few weeks after the administration of the chemical (*2*, *3*). There were extensive tubular necroses present. The tubular lumens were distended and filled with albuminous material, which not infrequently was dark blue in hematoxylin-eosin stained sections. Some of the tubules revealed regenerative epithelial changes in the form of dark-staining epithelium containing giant cells with hyperchromatic and irregularly shaped nuclei.

Remarks.—The sarcomas which developed in the right thigh of 11 rats of this group must be ascribed to a cancerigenic action exerted by the uranium deposited in the marrow cavity of the femur and the surrounding muscle tissue. Four of the 9 sarcomas formed metastases in the inguinal, abdominal and/or mediastinal lymph nodes and/or the lungs providing, thereby, incontestable proof of the malignant nature of the primary tumors. The majority of the sarcomas were spindle-cell sarcomas; 2 produced trabecular bone. While most of the tumors seemed to originate from the periosteum of the femur, the femur appeared to be uninvolved in 2 rats and these sarcomas obviously developed from the fibroblastic tissue surrounding the uranium deposits in the muscle tissue. The close relation of the sarcomas to one or several uranium foci was evident in every case, although there was often no direct contact of sarcoma cells with the uranium clumps. These usually were separated from the tumor tissue by a narrow-to-wide band of hyaline matter. Metastases were present in 6 cases, involving the lungs and the regional as well as mediastinal lymph nodes.

The 3 rats with acute nephrotic and calcinotic renal lesions displayed the typical picture of an acute and subacute uranium nephrosis, which

apparently was due to the escape of uranium from the site of injection into the circulation, resulting in the arrest of uranium particles in the pulmonary capillaries.

Experiment II. Intrapleural Injection of Metallic Uranium

Thirty-three rats, Osborne-Mendel strain, comprising 25 males and 8 females, approximately 5 months old, were injected through the right supraclavicular fossa into the pleural cavity with 0.05 cc. of a suspension of powdered metallic uranium in lanolin (25 percent uranium by volume, 60 percent by weight, 50 mgm. uranium per dose). The injections were repeated in monthly intervals until a total of 6 injections had been given. About one-half of the rats thus treated subsequently developed extensive loss of hair and excoriations of the skin. Six rats died during the first 6 months, 10 died during the next 6 months, and 17 died during the third period of 6 months. None survived more than 18 months after the start of the experiment.

Blood findings.—Blood examinations made once every 2 months for the first 6 months did not reveal any marked variations in the number of erythrocytes and leukocytes, in the differential count, or in the amount of hemoglobin, excepting that the number of eosinophilic leukocytes rose from 2.13 percent before the start of the treatment to 3.2 percent at the end of it. A final blood examination made 9 months after the first injection, on the other hand, showed a marked drop in the number of erythrocytes (from an average of 8,120,000 erythrocytes to 5,290,000) and in the amount of hemoglobin in 11 rats. This reduction ranged from one-third to less than one-half of the original value. There was at that time marked polychromasia and poikilocytosis present, indicating an active regenerative activity of the erythropoietic tissue. No significant changes in the leukocytes were noted.

Necropsy findings.—Soft black clumps attached to the pulmonary and thoracic pleura were found in the right pleural cavity. Additional black matter was present within the muscle tissue on the external side of the right thoracic wall. The right lung was adherent to the thoracic wall in a few cases. Bloody serous fluid was occasionally found in the pleural cavity. The lungs were often grayish red or dark red and indurated. The kidneys were swollen and had a mottled cortex, containing numerous small white spots. The testes were small, soft, and collapsed.

In 2 rats that survived for more than 12 months, tumors were found at the site of injection, *i.e.* the right side of the chest wall. One rat had an irregularly round mass of firm consistency, measuring 5 × 5 × 4 cm., which was attached to the ribs and extended upward and backward involving the shoulder and the spine. There were several black spots embedded in the superficial parts of the tumor. The tumor in the second rat consisted of multiple black nodules located in the right axillary region and measuring up to 0.5 cm. in diameter.

Encapsulated, nodular, soft, white tumors situated in the subcutaneous tissue of the mammary region were present in 3 additional rats. The

largest one measured 5 × 4 × 1.5 cm. None of them was adherent to the skin or chest wall. They had the typical location and texture of adenofibromas of the breast.

Histology.—Sternum, ribs and thoracic vertebrae, in all rats surviving for more than 6 months, showed distinct fibrotic and hyalinizing processes in the marrow, usually associated with a proliferation of trabecular bone. These changes were found whenever uranium was present in the periosteal tissue.

The chest wall contained, within the muscle tissue, many uranium deposits enclosed in a network of hyaline matter or fibrillar connective tissue. In addition to these usually multicystic formations, there were sometimes present small granulomatous foci containing phagocytes loaded with uranium dust. In one case, such deposits were surrounded by a highly cellular, fibroblastic tissue.

The 2 tumors located at the site of injection consisted of masses, sheets, and strands of large oval and spindle-shaped cells, with hyperchromatic nuclei, invading the surrounding muscle tissue. There were numerous mitotic figures as well as multinucleated giant cells in the larger of the two spindle-cell sarcomas. In both tumors uranium deposits were embedded in the sarcomatous tissue.

The 3 subcutaneous tumors contained glandular formations embedded in a fibrillar and hyaline connective tissue and, thus, were coincidental adenofibromas.

Purulent bronchitis, and lobular and lobar pneumonic changes were frequently seen. In several lungs, black uranium deposits were encountered within the pulmonary parenchyma. These deposits were surrounded by a thickened hyaline wall and by an area of fibrotic interalveolar septa. The uranium precipitations in the pleura were found in a loose, highly vascular, edematous matrix forming in many cases villous projections. Calcified plaques were frequently noted in the subendothelial space of the pulmonary artery. In a few rats, fibrous thickening of the intima of pulmonary arterioles occurred in the neighborhood of uranium deposits. The alveolar spaces near uranium deposits were filled with small oval- to spindle-shaped cells in one rat, while in a second rat numerous irregular, glandlike formations lined by a cuboidal epithelium were found.

A diffuse necrosis of the tubular epithelium of the kidney was present in most of the rats that died during the first 4 weeks of the experiment. In all other rats, there were numerous small or large cortical scars present, represented by a thickened interstitial fibrous tissue containing round-cell infiltrations. The tubular lumina were often cystically distended and filled with albuminous and sometimes calcified matter, while these and other tubules were lined by a dark-staining epithelium. In several rats, the destruction and reduction of the tubules had reached such a degree that there existed a remarkable crowding of the glomeruli due to the reduction of cortical tubules. Such kidneys resembled fetal kidneys in the number and distribution of glomeruli. The kidneys of several rats with advanced tubular changes also displayed some fibrotic and hyaline glomeruli.

Testes.—Conforming with the gross appearance of these organs in the majority of the rats, there was a complete or almost complete atrophy of the spermatogenic epithelium. The collapsed and empty tubules were located in an interstitial tissue showing focal proliferations of interstitial cells. In some of the testes with less advanced changes, there were spermatid giant cells and arrest of spermatogenesis.

Remarks.—The moderate-to-severe acute as well as chronic renal lesions and the distinct reduction in the number of erythrocytes that developed in an appreciable number of the rats after 6 injections of about 50 mgm. of uranium each, provide adequate evidence for the production of general toxic effects by the uranium metal administered. These reactions are identical with those previously described as effects of uranium poisoning by other investigators [Howland (*4*), Barnett and Metcalf (*2*), and Tannenbaum (*3*)]. The 2 sarcomas that developed in the right thoracic wall 12 months after the intrapleural injection of a total of about 300 mgm. of powdered metallic uranium, are evidently cancerous responses to the chemical agent. The close topographical relation of these sarcomas to the uranium depots, as well as the existence of hyalinizing and fibrotic changes with reactive cancellous bone proliferation in adjacent bones, support this interpretation.

Controls

Since sarcomas have been seen in the subcutaneous tissue of rats following the injection of various substances, mention may be made of a series of similar experiments in which asbestos, arsenic, chromium, and chromite suspended in lanolin, and lanolin alone, were injected in identical amounts into the femurs and pleural cavities of rats. Sarcomas were not found in any of the 150 rats injected intrapleurally, while 5 sarcomas developed at the site of injection among the 150 rats that received the chemicals into the marrow cavity of the femur. In recent experiments in which lanolin was replaced by a gelatin solution as a suspension medium for powdered nickel, rapidly growing sarcomas of the thigh developed in considerable number within 4 to 6 months after a single injection of such nickel-gelatin suspensions, while none so far has been observed among the gelatin controls. For these reasons, it is most unlikely that the vehicle played any significant role in the production of the sarcomas observed in rats following the intrafemoral and intrapleural injection of uranium and nickel.

COMMENT

The evidence presented leaves no doubt that parenterally introduced powdered metallic uranium, which was suspended in lanolin and deposited either into the marrow cavity of a femur or into a pleural cavity of rats, may produce systemic metallotoxic reactions. This effect was much more marked after intrapleural administration of a total of about 300 mgm. of uranium, given monthly in 6 equal doses of about 50 mgm. each, than after a single intrafemoral injection of 50 mgm. in the same vehicle, and

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was most strikingly demonstrable in the renal damage and the reduction of erythrocytes resulting. From the considerable amounts of uranium found at the sites of injection, however, it is obvious that these toxic effects are attributable to relatively small fractions of the original quantity administered, which gained access to the body fluids. The bulk of the injected material remained as a depot where it was placed.

The usually rather minor local degenerative changes seen in the tissues surrounding uranium depots, *i.e.* hyalinization and fibrosis, as well as the not infrequent occurrence of endosteal and periosteal thickening with cancellous bone production in the immediate neighborhood of such deposits, represent anatomical reactions which may be attributable to either a metallotoxic or a radiotoxic effect. The comparatively weak toxic action of uranium upon the tissues surrounding the depots might be explained by the fact that the small amounts of uranium dissolved from the depots combine with albumin and form strongly associated complexes with bicarbonates and with a number of organic acids [Barron (*5*)] and, thus, may be prevented from exerting a direct chemical effect on the neighboring cells. The low penetrating power of alpha rays, on the other hand, likewise might plausibly account for the rather narrow degenerative zones around the uranium depots.

These observations and considerations have a certain weight in determining whether the sarcomas which developed at the sites of injection of the uranium represent reactions of the tissues to certain chemical or physical effects of the uranium particles, or are the result of the emission of alpha radiation from the uranium depots. Recent experiments have shown that the parenteral administration of metallic nickel powder given to rats in similar amounts and in the same vehicle and at identical sites, caused the development of cancers in an appreciable number of these animals after an equal latent period [Hueper (*6*)]. In view of these observations, the possibility that the uranium sarcomas might represent another example of metal carcinogenesis had to be considered.

There is indeed a reasonably close relationship between the incidence rates of sarcomas among the rats of both experiments. This may suggest the operation of a causal mechanism similar in nature and intensity in both experiments (table 1).

It may be mentioned, moreover, that the cancers of the uranium series did not differ a great deal in their histologic structure from those observed in the nickel series. The only exception was presented by two bone-forming sarcomas among the uranium tumors, since none of this sarcoma type was present among the nickel cancers. However, such differences should not be taken too seriously, because they may be coincidental and without any etiologically specific significance. Both uranium and nickel, injected into the femur, produced local necrotizing, hyalinizing, and fibrosing reactions which not infrequently were associated with a local proliferation of periosteal and endosteal cancellous bone.

In support of a hypothesis that uranium-induced sarcomas may arise from a potential radiocarcinogenic agent, reference may be made to the

TABLE 1.—*Incidence of sarcomas among rats injected with uranium and nickel*

Route of injection	Metal	No. of rats	Rats dead within minimal latent period	Cancers at site of injection		Cancers of abdomen		Total number of cancers among survivors of 7–24 months	
				No.	%	No.	%	No.	%
Intrafemoral	Uranium	33	3	11	36	0	0	11	36
	Nickel	25	3	6	27	2	9	8	36
Intrapleural	Uranium	33	6	2	7	0	0	2	7
	Nickel	25	14	8	72	0	0	8	72
Both routes	Uranium	66	12	13	24	0	0	13	24
	Nickel	50	17	14	42	2	6	16	48

fact that the uranium was present in relatively large clumps, from the superficial layers of which comparatively concentrated local alpha-radiation effect was exerted over a long period of time on cells in their immediate vicinity. This factor perhaps deserves special mention because the intensity of exposure to alpha radiation from such foci is fundamentally different from the amounts and distribution of radiation which are present from uranium stored in the bones following its intravenous introduction, although the total quantity of uranium in the body in both conditions may be identical.

The length of the latent period of the uranium sarcomas is well within the limits of those given by Uehlinger (*7*) for sarcomas produced in rats by the action of radium (7 to 22 months) and thorium (10 to 27 months), as well as of those observed by Dunlap, Aub, Evans, and Harris (*8*) in rats following the feeding of radium (8 to 14 months).

The frequency of metastases noted in the uranium series (6 from 13 sarcomas) is even higher than that recorded by Dunlap, Aub, Evans, and Harris (2 from 9 osteogenic sarcomas). Since plutonium, a pure alpha-radiation emitter, readily produces cancer, uranium might be expected to have the same effect, although the intensity of its radiation is very low in comparison with that of plutonium. It may be well to mention in this connection that according to Evans and Goodman (*9*), rats and mice are about 40 times as resistant as humans to neoplastic reactions caused by alpha radiation, when all dosages are expressed in erg units.

These experimental observations may have some practical importance because of the unusually high frequency of cancer of the lung observed during the last 25 years among the uranium ore miners in Joachimsthal. Baader (*10*) recently reported that during the years 1939 to 1943 a total of 180 miners received compensation for cancer of the lung, and that there had been employed in these mines in 1929 a total of 323 miners.

A final decision of the question as to the exact nature of the carcinogenic action of uranium cannot be made from the evidence on hand, and would only be possible if a variety of uranium having no radiation could be

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tested for carcinogenic properties, or if entirely different criteria for distinguishing between the two causal mechanisms of metallocarcinogenesis and radiocarcinogenesis should become available. This problem, however, is mainly an academic one and of lesser importance, from the practical viewpoint, than the existence of potential cancer hazards to man occupationally exposed to uranium dust and fumes.

SUMMARY

1. The deposition of approximately 50 mgm. of uranium into the marrow cavity of a femur resulted in the development of 11 sarcomas at the site of injection among 33 rats, of which 30 survived the minimal latent period of 6 months.

2. The intrapleural administration of approximately 300 mgm. of uranium, given in 6 monthly injections, caused the appearance of 2 sarcomas originating from the chest wall among 33 rats thus treated.

3. Two of the 13 sarcomas were bone-forming, and 6 produced metastases in the inguinal, abdominal, or mediastinal lymph nodes and/or the lungs.

4. All sarcomas either surrounded uranium deposits or were found in the immediate vicinity of them.

5. It is uncertain whether the sarcomas were due to a metallocarcinogenic action of uranium or were caused by a radiocarcinogenic effect of this radioactive chemical.

6. The observations reported demonstrate that localized uranium deposits in the tissues of rats, creating a high concentration of uranium in circumscribed areas, and the prolonged action of the uranium in "nontoxic" or low-toxic quantities on the cells in the immediate vicinity of such foci, exert a definite carcinogenic effect.

7. None of the 13 sarcomas seemed to originate from the endosteum. The sarcomas appeared either to be of periosteal origin or to stem from mesodermal elements of the thigh muscle.

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PLATE 26

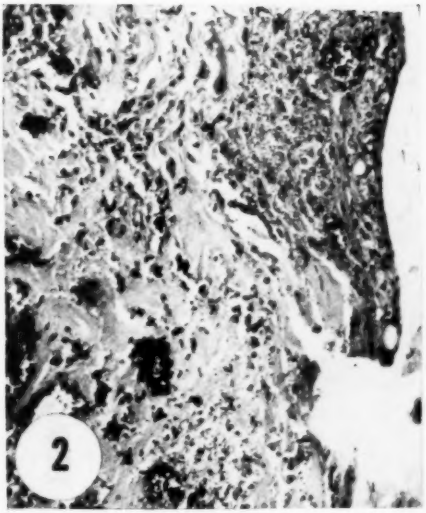
All figures × *185*

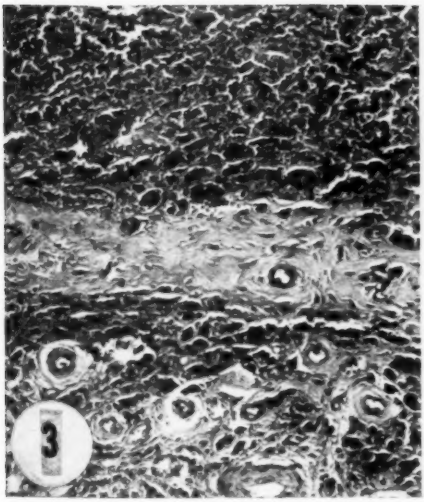
FIGURE 1.—X-ray photograph of tumor of rat #358 shows the deep shadows of uranium clumps in and near the large oval-shaped shadow of the tumor involving the femur, the pelvic girdle, and the spine.

FIGURE 2.—Uranium deposits in muscle tissue of the thigh, with extensive hyalinization of the tissues in their immediate vicinity. Formalin. Hematoxylin and eosin.

FIGURE 3.—Uranium granuloma composed of masses of phagocytic cells containing uranium "dust" particles. The walls of the small blood vessels within the granuloma are thickened. Formalin. Hematoxylin and eosin.

FIGURE 4.—Large and small uranium deposits in the femoral marrow, which is atrophic and in part hyalinized. Formalin. Hematoxylin and eosin.





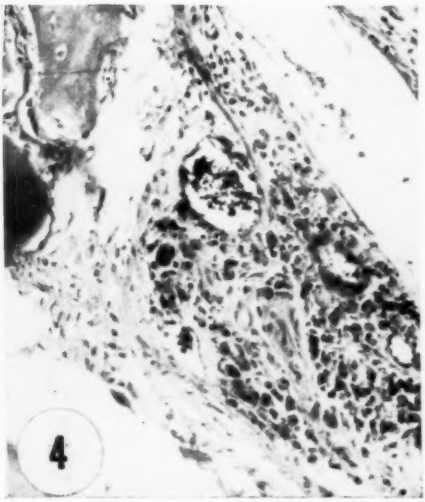


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PLATE 27

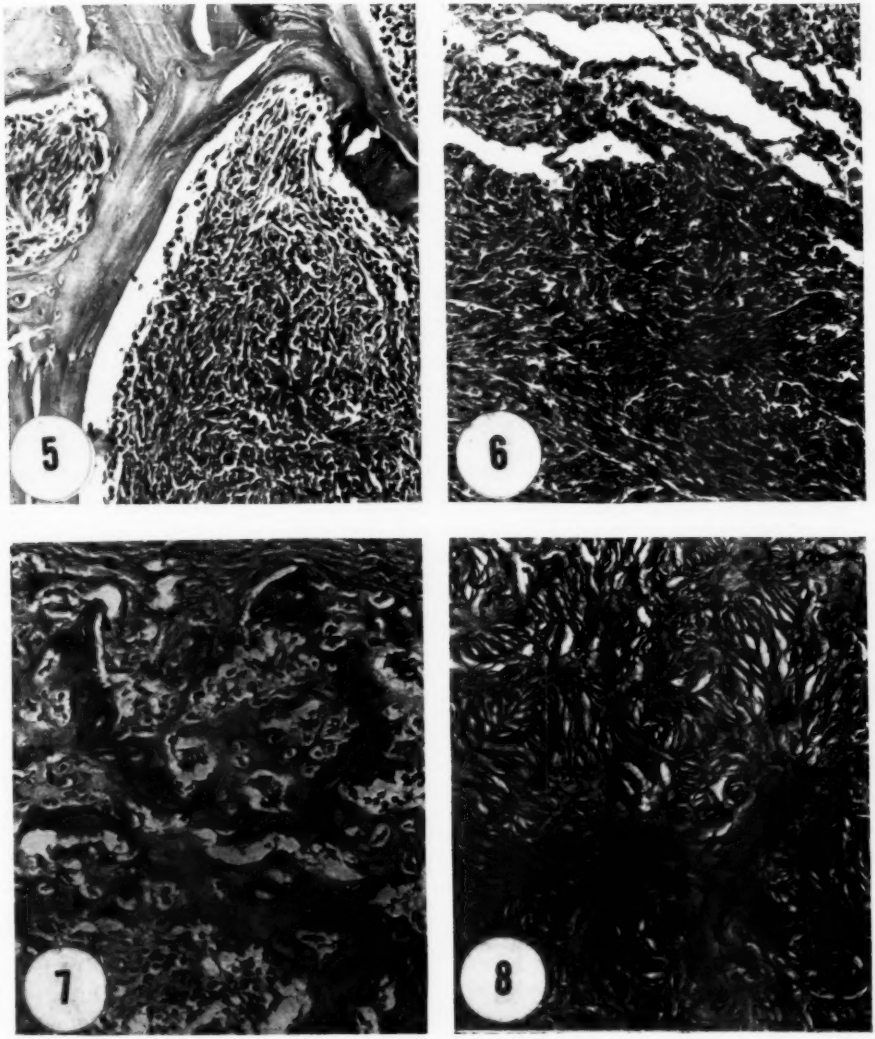
All figures × *185*

FIGURE 5.—Spindle-cell sarcoma showing invasion of the periosteal proliferations of cancellous bone. Hematoxylin and eosin.

FIGURE 6.—Metastatic spindle-cell sarcoma invading the lung tissue. Formalin. Hematoxylin and eosin.

FIGURE 7.—Periosteal bone-forming sarcoma. Formalin. Hematoxylin and eosin.

FIGURE 8.—Intra-abdominal metastases of sarcoma displaying masses of slender spindle cells often arranged in palisades, and hyaline areas with large nuclei. Formalin. Hematoxylin and eosin.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Influence of Sex Hormones on the Incidence and Form of Tumors Produced in Male or Female Rats by Gastric Instillation of Methylcholanthrene [1] [2]

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In previous experiments (*1*) it was found that methylcholanthrene, when introduced daily into the stomach over long periods of time, induced a high incidence of breast carcinoma in female Wistar rats. In male animals this chemical produced tumors less frequently, and the tumors were nearly all of spindle-cell variety (*2*). The present paper deals with the influence of sex hormones on the latent period, incidence, and type of tumors so produced.

MATERIALS AND METHODS

Random-bred male and female Wistar rats were divided into 14 groups of 25 animals each, and treated as indicated in table 1. Twenty-five male and twenty-five female rats, not listed in table 1, were retained as untreated controls. Each animal was housed singly in a cage with wide-mesh bottom. All animals were given Rockland diet and tap water *ad libitum*. Methylcholanthrene (2 mgm.) in olive oil (0.5 cc.) was instilled daily into the stomach of each treated rat through a rubber catheter tube until the animal died or was sacrificed.

All males were started on treatment when weighing 60 to 80 gm. and at an age of 30 to 60 days, when the majority could be considered immature (*3*). Females were divided into two groups: in one group the animals weighed 60 to 80 gm., the vagina being as yet closed; in the second group the treatment was not begun until after the estrus cycle had developed. Thus, animals of groups F2, F6, and F8 (table 1) weighed 110 to 130 gm. and were 60 to 90 days old when the treatment began.

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[2] The authors wish to express our appreciation to Professor Robert H. Peckham of the Department of Research Ophthalmology, Temple University, for his work on the statistical evaluation of the paper and for his constructive criticism. It is a pleasure to thank Dr. Harold L. Stewart and Dr. Thelma B. Dunn of the National Cancer Institute, for valuable suggestions during the development of the work.

[3] Fellow of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

TABLE 1.—*Methods of treatment of rats with sex hormones and methylcholanthrene*

Group No.*	Condition of animals	Hormone implanted	Weight at beginning of treatment (*grams*)
		MALES	
M1	Intact		60–80
M2	Intact	Estradiol 25 mgm.	60–80
M3	Intact	Estradiol 25 mgm †	60–80
M4	Intact	Estradiol 25 mgm †	60–80
M5	Castrated	Estradiol 25 mgm.	60–80
M6	Castrated		60–80
		FEMALES	
F1	Intact		60–80
F2	Intact		110–130
F3	Intact	Progesterone 50 mgm.	60–80
F4	Intact	Testosterone 37 mgm.	60–80
F5	Intact	Testosterone 75 mgm.	60–80
F6	Intact	Testosterone 37 mgm.	110–130
F7	Spayed		60–80
F8	Spayed		110–130

*All groups except M2 received methylcholanthrene.
†The difference between groups M3 and M4 is that in M3 methylcholanthrene was started and an estradiol pellet was implanted at the body weight of 60-80 gm., whereas in M4 the methylcholanthrene was started at the body weight of 60–80 gm. but the estradiol was implanted 4 months later.

The difference in age between animals weighing 60 to 80 gm. and those weighing 110 to 130 gm. varied between 30 and 60 days. To test maturity in female rats, the estrus cycle was determined by the vaginal smear method. In spayed females, the cycle was determined before spaying, and vaginal smears were repeated after spaying to establish the absence of the cycles.

Hormones [4] were implanted subcutaneously at the time treatment with methylcholanthrene was begun, except for the animals of group M4, which received implants of estradiol 4 months after the initial treatment with methylcholanthrene.

Most of the rats were sacrificed when they appeared ill, or when the tumors became so large that they interfered with respiration or other vital functions. Samples of all tissues were taken during ether anesthesia, or immediately after death. All animals found dead in their cages were autopsied and specimens of tumor and organs showing gross pathologic alteration were taken for microscopic examination. All tissues were fixed in Zenker's acetic acid fluid or in 10 percent neutral formalin, and sections stained with hematoxylin and eosin. Phosphotungstic acid-hematoxylin for myofibrils, toluidine blue for mast cells, Sudan IV for fat, and Foote's modified silver impregnation for reticulin were also utilized for some tissues.

[4] The authors are indebted to Dr. W. H. Stoner of the Schering Corporation for the hormones, the composition of which was as follows: progesterone in 50 mgm. tablets; testosterone (Oreton F) in 75 mgm. tablets; and estradiol (Progynon) in 25 mgm. tablets.

At the outset of the experiment, each group comprised 25 rats, but because of animal fatalities during a laboratory accident, the experiment was finished with fewer rats than had been anticipated. Except for those of group F1, all animals that died at 175 days of age or less were excluded from the data; because of the early appearance of tumors in animals of group F1, only those that died before 100 days were excluded. In the statistical evaluation, the Chi square test was used to solve double dichotomies, and all probabilities are expressed as percentages.

RESULTS

Pathology of Induced Tumors

We have designated five categories into which the tumors fall: glandular mammary tumors, spindle-cell mammary tumors, fibroadenoma of the breast, collagenous tumor of the breast, and mesenteric sarcoma.

Tumors classified as glandular mammary tumors seemed to originate in a focal group of dilated ducts of the dermis (fig. 1); in many cases the duct lumen was filled with acidophilic amorphous material. Subsequently, the walls of many of the ducts showed evidence of proliferation, so that most of the ducts were lined with two or three layers of small cuboidal cells which resembled cords of cells that appeared to have encroached upon the lumen of the duct, partially or completely obliterating it (fig. 2). In this growth the basement membrane was still intact and covered internally by a layer of spindle-shaped myoepithelial cells (fig. 3). In more advanced tumors showing evidence of proliferation, the myoepithelial cells were cuboidal (fig. 4). At this stage of development, many of the ducts were found to be in the form of cords and islands of tissue greatly resembling basal-cell carcinoma or cylindroma (fig. 5). Histologically, these tumors appeared to be benign, similar to a basal-cell carcinoma; however, they grew rapidly when transplanted into the subcutaneous tissue of female rats less than 7 days old.

In older tumors, squamous-cell metaplasia was seldom found, although when it did occur it was usually adjacent to an area in which the tumor was necrotic, due to ulceration and infection. Vacuolation of cells occurred in various areas of some tumors, and in other instances it was evident that proliferation had established large groups of juxtapositional glands. The intracellular vacuoles stained uniformly with Sudan IV in frozen sections. The glandular cells of the tumor sometimes invaded adjacent tissues such as skeletal muscle (fig. 6). Metastases to regional glands, pleura, and lung (fig. 7) seldom occurred. At this stage of growth, the tumor cells were anaplastic, usually having deeply colored nuclei and numerous mitotic figures. Certain cases exhibited absence of glandular architecture, or even absence of solid circular epithelial masses such as those existing at earlier stages of growth. The epithelial cells were elongated and appeared as strands emanating from the gland to become mixed with the stroma as spindle-shaped epithelial cells (fig. 8).

During the earliest stage of tumor formation, the stroma may be scant. The glandular elements were in some cases surrounded only by fat. At

later stages of development (fig. 2), however, the stroma was composed of collagenic fibers interspersed with thin elongated cells. In normal glands, the intralobular stroma is usually derived from myoepithelial cells, whereas the stroma of many tumors in this series appeared to have been derived at least in part from myoepithelial cells.

The fibroadenomas were usually composed either of acini or of ducts which appeared to be compressed by stroma. The duct cells were apparently quiescent, and the stroma consisted of compact collagenic fibers in which a few nuclei could be seen (fig. 9). Gross examination revealed fibroadenomatous tumors to be firm, and difficult to cut because they yielded to pressure of the knife. The lobules were separated by sheets of fascia.

Tumors classified as collagenous tumors of the breast usually possessed few cells, being composed chiefly of thick compact collagenic lamellae similar to that seen in figure 9. These tumors were hard, milk white in color, and possessed a gritty consistency.

The sarcomas formed in the mesentery and mesenteric lymph nodes appeared as wild anaplastic growths with numerous multipolar mitotic figures. Giant cells were common and the adjacent structures were invaded by tumor cells (figs. 10, 11). They were firm tumors containing many foci of necrosis.

The spindle-cell tumors of the breast proved to be widely variable in degree of morphologic differentiation. The more anaplastic ones resembled the mesenteric sarcomas described above (fig. 12), while other tumors had a more orderly arrangement, being composed of whorls of spindle-shaped cells, the nuclei of which were all the same. Portions of these tumors structurally resembled neurofibromas and leiomyomas (figs. 13, 14).

Stromatization of Myoepithelial Tissue Under the Influence of Sex Hormones

The authors have observed three histologic structural states that seem to demonstrate the stromatization of epithelial elements. In the first, the glandular elements were initially carcinomatous, with small duct-lining cells strung out like beads on fragments of disorganized basement membrane (fig. 15).

Figure 7 shows two pulmonary metastases of an adenocarcinoma of the breast. Figure 16 shows the upper metastatic lesion, which varies from the original adenocarcinoma (fig. 17) in that it possesses the characteristics of a stromal type of tumor.

In a second possible method of transformation to spindle-cell tumors, the tumor appeared to have failed to pass through a carcinomatous phase. In its earliest stage, it presented a whorled appearance. The whorled tissue was originally believed to be sarcomatous and probably of mesodermal origin (*2*), but after studying many of these tumors it was concluded that they derive from the myoepithelial cells of a fibroadenoma. The epithelial cells may at first perhaps have undergone a change resembling that leading to atrophy, but it is doubtful whether they disappear.

Neither Warburton and Reece (*3*) nor the present authors have found evidence of a mechanism that could be interpreted to provide phagocytosis of cellular debris. At the beginning of the dedifferentiation process, the epithelial cords were more and more narrow, and the cells more oval and spindle-shaped (figs. 18, 19). These cells were colored less intensely with hematoxylin and eosin. At a later stage, the cells assumed a whorled pattern, and as at earlier stages of treatment surrounded a central group of cells that represented the disappearing gland. In some cases, the lumen of a duct could be seen. This whorled structure possessed all the features of a sarcoma with elongated, thin cells, the nuclei of which appeared simply as dark streaks in spindle-shaped cytoplasm (fig. 20).

Although the formation of cartilage was never found, one case exhibited bony structure. Tissues of other rats revealed nodules composed of loosely packed giant cells. These bizarre giant cells possessed many cytoplasmic processes. The nuclei were often represented by triangular clumps of dark-staining material (fig. 21), and unusual forms of mitotic figures were present. Portions of many of these tumors may resemble cystosarcoma phyllodes, and in general should be regarded as mixed tumors of the breast.

A third type of metaplasia is illustrated in figure 22, in which stellate stromatization of epithelial tissue seems to have taken place. Previous writers have said that the cartilage-like material in mixed tumors (*4*, *5*) is derived from epithelium. Despite the lack of cartilage in the tumor shown in figure 22, it appears to represent an early stage of mixed tumor, prior to the disappearance of epithelial cells.

The present observations agree with Allen's (*4*) and others' (*6–11*) views that the morphologic variation is the result of metaplasia of epithelial cells, and the capacity of the myoepithelial cell to transform into another type of cell. Since the type of tumor produced is dependent upon the predominance of one or the other of male or female sex hormones, the direction of transformation of the myoepithelial cell into glandular or stromal form also depends upon the sex hormone balance.

Changes Produced in Other Organs by Sex Hormones

Female rats treated with testosterone developed hypoplasia of the pituitary gland, of the adrenal gland, and of the genital organs. Male rats treated with estradiol revealed hyperplasia of the prostate gland. Estradiol apparently induced hypertrophy of the adrenal gland in male rats, and atrophy of the glandular portion of the prostate gland with overgrowth of its fibromuscular elements. Spermatogenesis was inhibited with resultant atrophy of the seminiferous tubules.

The pituitary gland of the male rat treated with estradiol alone, or with estradiol and methylcholanthrene, was enlarged and purple in color, resembling in gross and microscopic characteristics such pituitary glands first described by Cramer and Horning in 1936 (*12*). The glands were enlarged in all of the rats so treated. In one animal, the tumor extended

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laterally inducing vestibular symptoms. Microscopic examination revealed large cysts filled with red blood cells, surrounded by dense collections of chromophobe cells. After treatment with methylcholanthrene alone, no enlargement of the pituitary gland was found.

Incidence and Types of Tumor Produced

Table 2 shows the results of treatment for each experimental group of animals. It should be noted that certain animals developed more than one tumor, although text-figure 1 shows the time of discovery of only the first tumor in each animal. Since the graph is cumulative, any portion of a line with slope zero between two points represents no increase in the number of tumor-bearing animals in that period of time, but does not imply that an animal already having developed one tumor is not developing others.

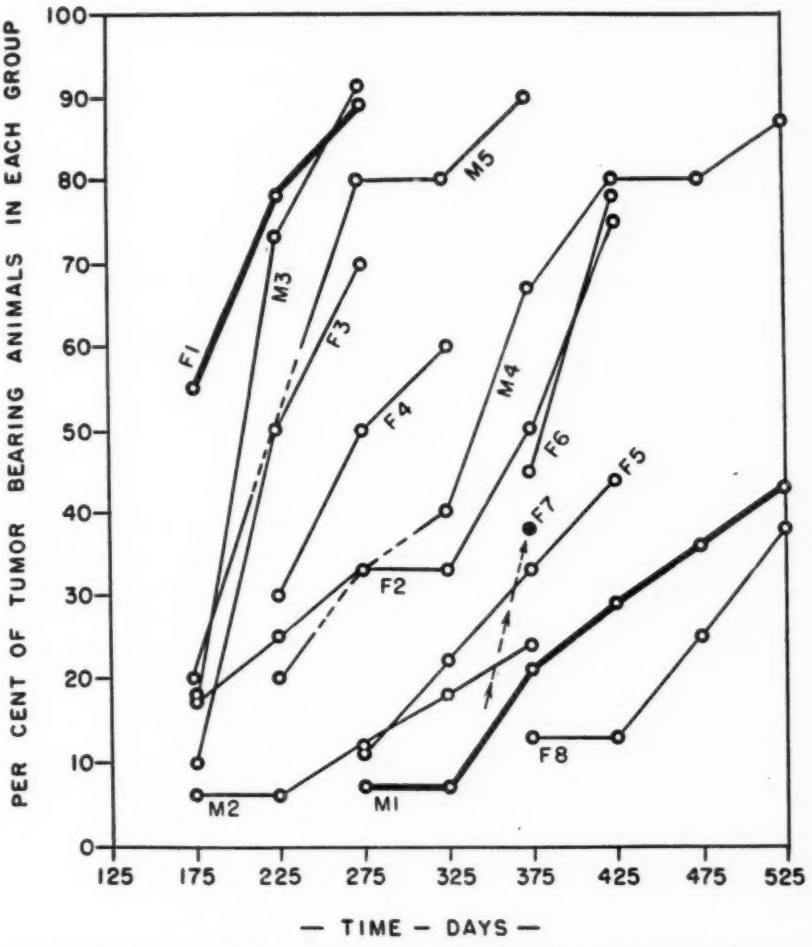
Table 2.—*Data on tumors for all experimental groups*

Group No.	Number of rats in groups	Number of rats having tumors	Number of rats having multiple tumors	Total number of tumors	Number of each type of tumor					Average age of animals when first tumor appeared (*days*)
					Glandular tumors of the breast	Spindle-cell tumors of the breast	Mesenteric sarcoma	Fibroadenoma of the breast	Collagenous tumors of the breast	
M1	14	6	2	8	1	4	2	1	0	386
M2	17	4	0	4	3	0	0	0	1	301
M3	11	10	6	21	19	1	0	1	0	226
M4	15	13	8	27	12	1	1	12	1	332
M5	10	9	6	19	15	1	0	3	0	245
F1	18	16	12	37	36	1	0	0	0	195
F2	12	9	3	15	11	0	0	4	0	313
F3	10	7	4	12	11	0	0	1	0	231
F4	10	6	4	15	12	0	0	2	1	263
F5	9	4	1	9	6	0	1	0	2	367
F6	9	7	4	12	5	3	3	1	0	401
F7	8	3	0	3	2	0	0	0	1	384
F8	8	3	0	3	1	1	0	0	1	466

In text-figure 1, the response of males and females is represented in groups M1 and F1. Although line F8 falls beyond line M1, we have arbitrarily chosen line F1 and M1 to represent the limits of male and female susceptibility to tumor formation shown by unaltered immature rats treated with methylcholanthrene.

A comparison of lines M2 and M1 shows the effect of estradiol alone (M2) in relation to the effect of methylcholanthrene alone (M1) in males. In groups F1, F4, and F7 treatment was started *before* the vagina opened whereas in three parallel groups (F2, F6, and F8) treatment was started *after* the vagina opened.

The effect of castration on tumor formation in female rats may be seen by comparing groups F7 and F8, which were spayed before methylcholanthrene treatment was started, with groups F1 and F2, treated only with methylcholanthrene.



TEXT-FIGURE 1.—Comparison of tumor incidence of all experimental groups of animals.

The effect of estradiol on the rate of tumor production and the number of tumors produced by methylcholanthrene in male rats may be seen by comparing groups M3, M4, and M5 with the limits of male and female susceptibility (M1 and F1).

Groups F3, F4, F5, and F6 represent the effects of testosterone or progesterone on the rate of tumor production and the number of tumors produced by methylcholanthrene in female rats.

The effect of estradiol on tumors produced in male rats by methylcholanthrene (groups M3, M4, and M5) can be compared to the effect of testosterone on the tumors produced by methylcholanthrene in female rats (groups F4, F5, and F6).

Group F2 shows the effect of maturity on the tumor-producing qualities of methylcholanthrene in female rats as compared to the effects of testosterone and progesterone on the tumor-producing qualities of methylcholanthrene in immature female rats (groups F3, F4, and F5).

Table 3 lists the data from which the graph was drawn.

TABLE 3.—*Percentages of rats in experimental groups developing tumors during each indicated time interval*

Days	M1	M2	M3	M4	M5	F1	F2	F3	F4	F5	F6	F7	F8
0–150	0	0	0	0	0	0	0	0	0	0	0	0	0
150–200	0	6	18	0	20	55	17	10	0	0	0	0	0
200–250	0	6	73	20	50	78	25	50	30	0	0	0	0
250–300	7	12	91	33	80	89	33	70	50	11	0	0	0
300–350	7	18		40	80		33		60	22	0	0	0
350–400	21	24		67	90		50			33	45	38	13
400–450	29			80			75			44	78		13
450–500	36			80									25
500–550	43			87									38
Number of animals with tumor	6	4	10	13	9	16	9	7	6	4	7	3	3
Total number of animals in group	14	17	11	15	10	18	12	10	10	9	9	8	8

In table 4 the various tumors that developed in the experimental animals are divided into three classifications: the glandular tumors of the breast, the fibroadenomas, and a third group comprising collagenous tumors of the breast, spindle-cell tumors of breast, and mesenteric sarcomas. It may be seen from table 4 that glandular tumors of the breast occur chiefly in animals in which the female sex hormone predominates, while the number of such glandular tumors is very small in rats having more male sex hormone than female sex hormone (compare groups F3, F4, F5, and F6 with their respective control groups F1 and F2). Similarly, the right-hand column of table 4 shows an increase in spindle-cell tumors in certain female rats due to an increase in male sex hormone. The

TABLE 4.—*Occurrence of different types of tumor in rats having different sex hormone status*

Group	Glandular tumors of the breast	Fibro-adenomas	Sarcomas and fibromas of the breast, and mesenteric sarcomas
M1	1	1	6
M2	3	0	1
M3	19	1	1
M4	12	12	3
M5	15	3	1
F1	36	0	1
F2	11	4	0
F3	11	1	0
F4	12	2	1
F5	6	0	3
F6	5	1	6
F7	2	0	1
F8	1	0	2

TABLE 5.—*Comparison of number of animals developing one tumor with number of animals developing multiple tumors*

Group	Total number of animals	Number of animals with tumors	Number of animals with more than one tumor
M1	14	6	2
M2	17	4	0
M3	11	10	6
M4	15	13	8
M5	10	9	6
F1	18	16	12
F2	12	9	3
F3	10	7	4
F4	10	6	4
F5	9	4	1
F6	9	7	4
F7	8	3	0
F8	8	3	0

largest number of fibroadenomas occurred in rats in which neither male nor female sex hormones predominated. The experiment seems to indicate therefore, that glandular tumors of the breast are more likely to occur when the female sex hormone predominates, that fibroadenomas occur when a balance between the physiological action of male and female sex hormones is achieved, and that all other tumors are due to the presence of excessive quantities of the male sex hormone.

Table 5 shows that if more than half the animals in a group developed tumors, 50 percent or more of the tumor-bearing animals bore multiple tumors. The likelihood of this finding being accidental is very remote, since when the double dichotomy is solved by the Chi square test, P is less than 0.01. Therefore, the probability that a tumor-bearing animal will bear multiple tumors will be greater if the animal comes from a group in which 50 percent or more of the animals bore tumors.

DISCUSSION

The data show that, although methylcholanthrene produces tumors in both male and female rats, the susceptibility of the animal to tumor formation and the type of tumor produced depend at least in part upon the sex of the animal. Susceptibility of a group of animals to tumor formation is indicated by the length of the latent period before tumors occur and by the percentage of animals in the group that develop tumors. Text-figure 1 shows that female rats treated with methylcholanthrene (F1) are more susceptible to tumor formation than male rats treated with methylcholanthrene (M1), and that all other groups [except spayed females (F8)] treated with methylcholanthrene in this experiment fall between the limits of susceptibility set by groups F1 and M1. Spayed mature females treated with methylcholanthrene (F8) appear to be even less susceptible to methylcholanthrene than normal males. A line drawn equidistant from, and roughly parallel to, the lines representing groups F1 and M1, would theoretically represent a group of animals in which

the susceptibility to tumor formation is dominated by neither the male sex hormones nor the female sex hormones. These animals, therefore, upon treatment with methylcholanthrene, would exhibit a response to carcinogens that would indicate a balance between the influence of male and of female sex hormones. The groups whose cumulative percentage lines fall between this theroretically *hormonally balanced* group and F1 are groups with a *female sex hormone dominance*, while those groups falling between the *hormonally balanced* group and M1 have a *male sex hormone dominance* in regard to production of tumors by methylcholanthrene.

Sex hormones also influence the type of tumor produced, glandular tumors of the breast developing most frequently in the female or female-hormone treated rat, while spindle-cell tumors, collagenous tumors, and mesenteric sarcomas predominate in the male or male-hormone treated rat. The fibroadenoma, a tumor with both glandular and spindle-cell components with neither component predominating, characteristically develops in hormonally balanced rats. Comparing table 2 with text-figure 1, it can be seen that groups M4 and F2, which approach the theoretically hormonally balanced group of rats in their susceptibility to tumor induction by methylcholanthrene, showed the highest percentage of fibroadenomas. This condition is most striking in group M4, the susceptibility line of which is nearly equidistant from those of groups F1 and M1 and in which 12 of 27 tumors (44 percent) were fibroadenomas.

It is believed that this is the first report describing the influence of sex hormones on the tumorigenic action of methylcholanthrene in rats. This influence has probably not been noted previously because the method by which the carcinogen is generally applied would not reveal this phenomenon. When relatively large amounts of methylcholanthrene are repeatedly fed to lactating rats by stomach tube, only minute amounts of the administered dose are subsequently found in the mother's milk (*13*). This implies that the material passes through the intestinal wall and into the blood stream, and thence to the tissues. In this way, tumors will develop first in those tissues most sensitive to the effect of methylcholanthrene. The presence of estrogens, which are tumorigenic when administered repeatedly in large doses (*14*), either sensitizes the breast tissue to methylcholanthrene, or works synergistically with it. Other sex hormones, such as testosterone and progesterone, interfere in some way with the tumorigenic action of methylcholanthrene. It cannot be said at this time how a possible alteration of the action of methylcholanthrene may occur. If, however, methylcholanthrene is applied locally, as by injection or painting, the high concentration of carcinogen may surpass the tumorigenic threshold and mask any synergistic or antagonistic effect that might be exerted by other substances.

When methylcholanthrene is instilled by stomach tube daily into the stomach of Wistar rats, three different tissues—*viz.*, breast tissue, fibrous tissue, and hematopoietic tissue—respond to it by proliferation (*1*, *2*, *15*). There is no ready explanation for this selective response, but the structural similarity between methylcholanthrene and the steroids, as well as the

responsiveness of these three tissues to adrenal cortical steroids and sex hormones may be of significance in this respect.

Astwood, Geschickter, and Rausch (*16*) found that until the age of 6 weeks there is no difference in development of the breast of male, female, and castrated rats. In castrated rats, growth of breast tissue stops at 6 weeks and involution occurs at 16 weeks of age. In 8-week-old female rats, the influence of the ovarian follicular hormones on the mammary gland is apparent. Whether this influence is a direct action, or an action mediated through the pituitary gland (*16*, *17*), it induces rapid extension of the duct system until maturity, which is signalled by the opening of the vagina. Most authors are agreed that estrogens cause extension of the duct system, but find a marked variation in different animals in regard to the cause of formation of acini (*18*). Ancel and Bouin (*19*), later supported by Maeder (*20*) and others, showed that progesterone, testosterone, and gonadotrophin can cause formation of acini in intact females. Selye (*21*) demonstrated formation of acini in castrated albino rats given large doses of progesterone alone, although smaller doses must be accompanied by estrogen to achieve the same effect.

Astwood and his associates believed that the proliferative changes of normal mammary gland tissue are closely related to the phases of the estrus cycle, and that after estrus, duct-buds and mature ducts become narrow. Large amounts of estrogen stunt the growth of the duct system and cause cysts to form (*16*, *22*), whereas large amounts of progesterone or testosterone (*16*, *21*) cause the development of acini. There is little evidence concerning possible variation of stroma induced by sex hormones.

The present experiments have demonstrated that male rats given methylcholanthrene develop a completely different type of tumor, compared with the tumor produced in female rats given the same substance. Tumors produced by other investigators with estrogen (*14*) are similar to the tumors found in female rats given methylcholanthrene and in male rats in which pellets of estradiol were implanted. Male rats given methylcholanthrene developed spindle-cell tumors, while the largest number of fibroadenomas occurred in the theoretical hormonally balanced group (M4). Progesterone at the dosage employed (F3) had no influence on the form of tumor developed. It may be concluded therefore that testosterone and estradiol, having different effects on breast development, also exert a determinative influence on the type of tumor that is to be produced by methylcholanthrene.

A number of authors have noted that transplantable fibroadenomas seem to lose their glandular structure when transplanted into males, and furthermore, that estrogen enhances the retention of the adenomatous character of these tumors (*6*, *24*). The results of the present experiments support this concept, and it is suggested that the fibrous and epithelial tumors do not represent fundamentally different tissues but rather that they are different forms of the same tissue component, *viz.*, the myoepithelial cell.

The epithelial origin of many types of tissue, including bone, cartilage,

and sarcomatoid tissue, found in cystosarcoma phyllodes and mixed tumors of the salivary glands, has been postulated by a number of authors. This concept has been recently restated by Allen (*4*).

All of the breast tumors produced in the present experiments are considered to be varieties of mixed tumors, and the relative amounts of estrogen and testosterone present determine the final structural pattern of these tumors. The fibroadenoma represents an indeterminate response to a hormonal balance, and it is believed that had estrogen prevailed in the rat, a glandular instead of a fibrous tumor would have formed. If testosterone had been the predominant hormone, a spindle-cell tumor would have formed.

One of the most significant results of these experiments is the marked decrease in incidence of glandular tumors in group F2 compared with that in group F1, and the tendency in the female rat to develop fibroadenomas when treatment with methylcholanthrene was delayed until maturity. Astwood and his associates state that in the rat immediately before the vagina opens there is a period of follicle stimulation resulting in the production of estrogen which in turn causes proliferation of epithelial tissue in the mammary ducts. It is possible that methylcholanthrene administered after this initial output of estrogen has become intermittent, when estrus has been established, induces fibroadenomas instead of glandular tumors because of the lack of continuous estrogen stimulation and the slight masculinizing effect of the progesterone that is produced during part of each cycle.

SUMMARY

Gastric instillation of methylcholanthrene into the stomach of Wistar rats over long periods of time resulted in the production of three fundamental types of tumor: glandular tumor of breast (predominating in female animals or in male animals given estradiol); spindle-cell and collagenous tumors of the breast and mesenteric sarcomas (predominating in male rats, or in female rats given testosterone); and fibroadenomas, *i. e.*, mixed tumors of the breast (predominating in spayed or castrated animals in which the sex hormone effects were experimentally counterbalanced). The susceptibility of the animals to induction of tumors by methylcholanthrene varied in the three groups of animals. It was most marked in female rats, least in the male rats, and fell between these two limits in rats having experimentally counterbalanced sex hormones.

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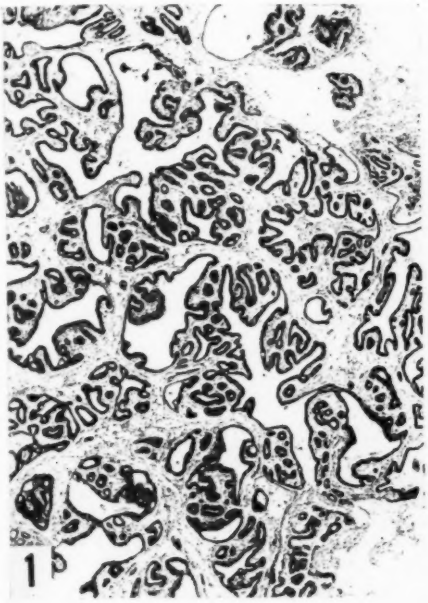
PLATE 28

FIGURE 1.—Early phase in the development of a glandular tumor of the breast. Note tortuous and dilated ducts. The epithelial lining of ducts is thin. Papillary projections may be seen to be extended into the lumen of some ducts. The stroma is loosely packed. Hematoxylin and eosin. × 35

FIGURE 2.—An apparently later stage of the same tumor as shown in figure 1. Increased proliferation of lining cells of the ducts occludes the lumina. Proliferation of stroma is present. The stroma contains numerous cells due to chronic inflammation. Hematoxylin and eosin. × 35

FIGURE 3.—Sold epithelial cord surrounded by a layer of spindle-shaped myoepithelial cells (arrows). Hematoxylin and eosin. × 250

FIGURE 4.—Another section of the tumor shown in figure 3, demonstrating cuboidal basal myoepithelial cells. Hematoxylin and eosin. × 250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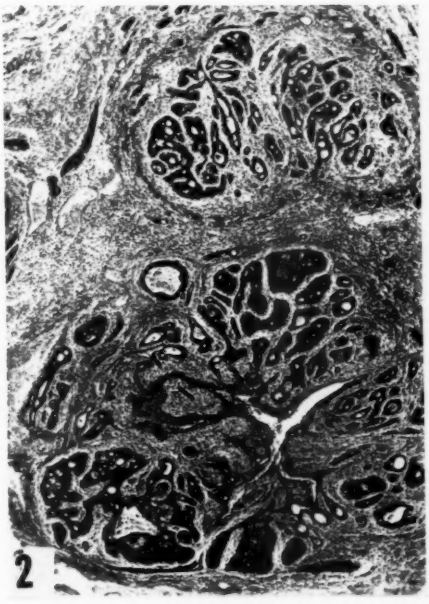
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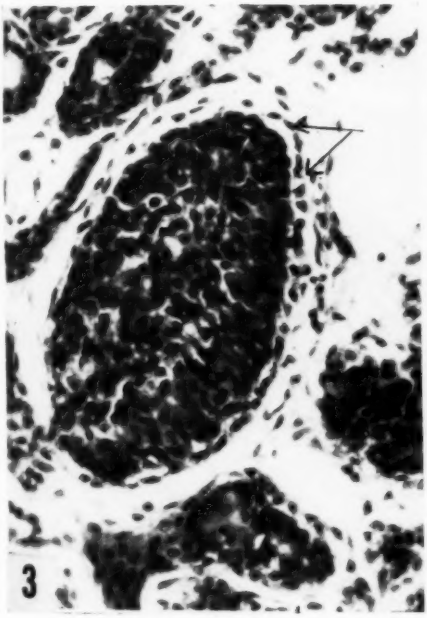
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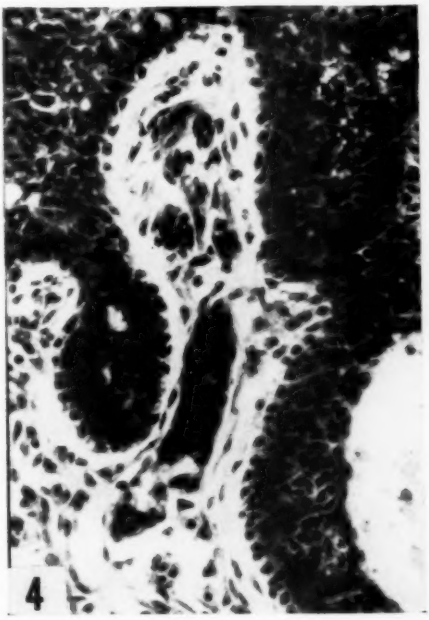
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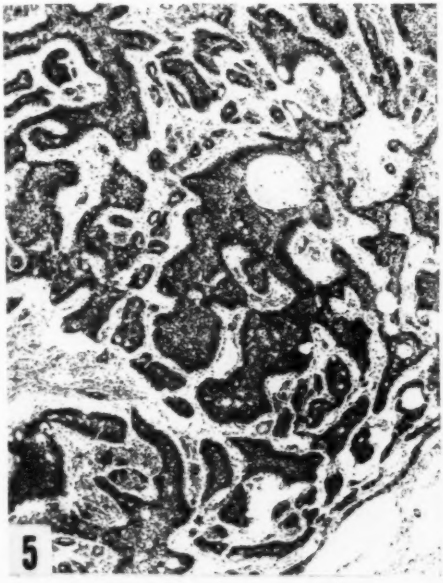
PLATE 29

FIGURE 5.—Low-power view of the tumor shown in figures 3 and 4, demonstrating its similarity to basal-cell tumors. Hematoxylin and eosin. × 35

FIGURE 6.—Glandular cells of adenocarcinoma of the breast showing invasion of adjacent striated muscle tissue. Hematoxylin and eosin. × 250

FIGURE 7.—Two forms of pulmonary metastases from adenocarcinoma of the breast. Hematoxylin and eosin. × 17



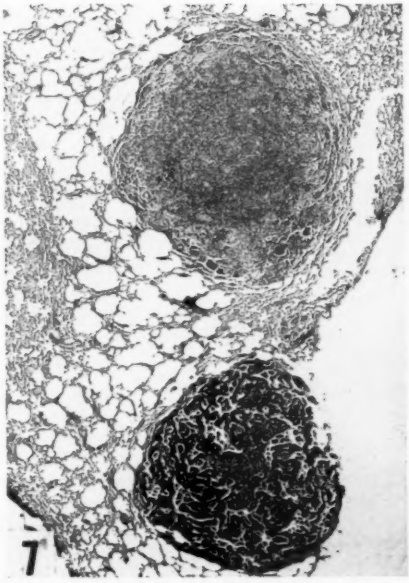


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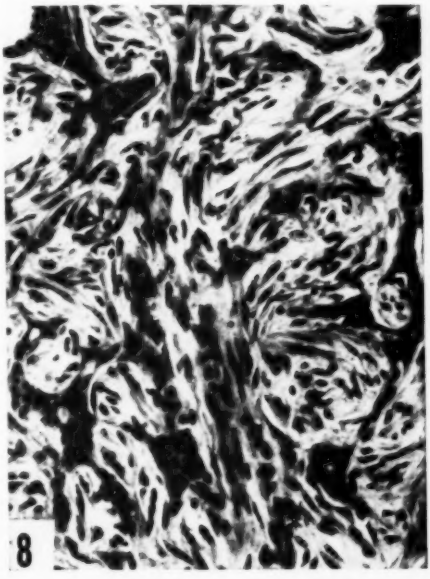
PLATE 30

FIGURE 8.—"Stromatization" of narrow epithelial cords in a glandular tumor of the breast. × 300

FIGURE 9.—Fibroadenoma of breast showing evidence of sclerosis. Note atrophic glands, and the dense collagenic fibers having few cells. × 200

FIGURE 10.— Mesenteric sarcoma showing invasion of the wall of the cecum. × 35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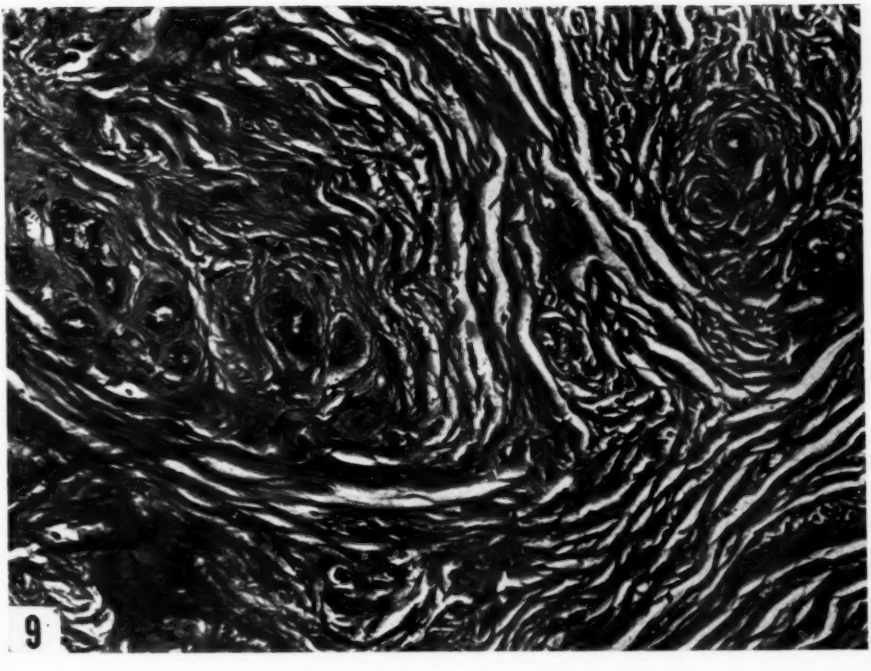
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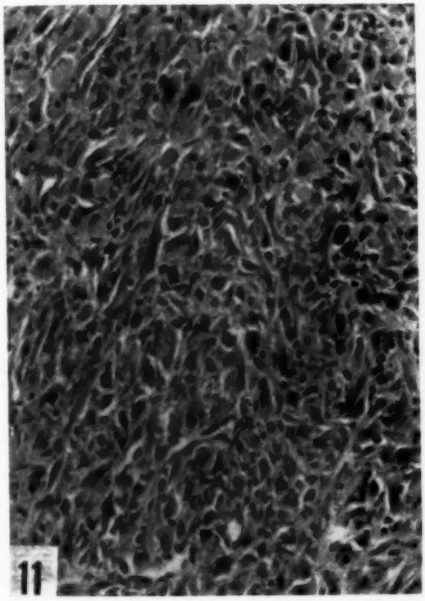
PLATE 31

FIGURE 11.—Higher-power view of mesenteric sarcoma shown in figure 10. × 250

FIGURE 12.—Spindle-cell sarcoma of breast, showing evidence of marked anaplasia and hyperchromatism. × 440

FIGURE 13.—Whorled spindle-cell myoepithelial sarcoma. Fat adjacent to a fibroadenoma completely infiltrated with sarcoma cells. The stroma of the fibroadenoma cannot be differentiated from that of the adjacent tumor. × 35

FIGURE 14.—Spindle-cell myoepithelial sarcoma shown in figure 13. × 450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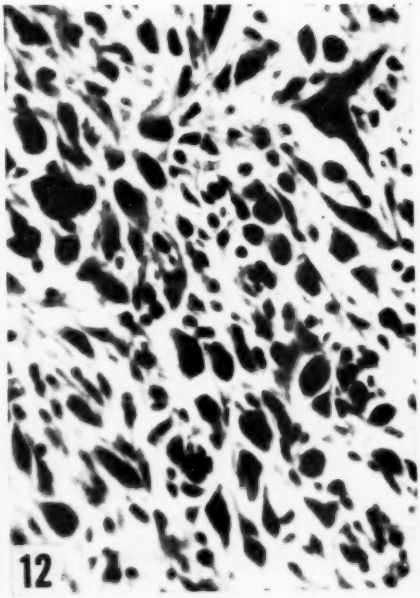
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PLATE 32

FIGURE 15.—Ruptured and elongated basement membranes, demonstrating the parallel arrangement of their fibers. Silver impregnation. × 250

FIGURE 16.—One of the pulmonary metastases shown in figure 7. This metastatic tumor appears to have been derived from an adenocarcinoma of the breast that had undergone "stromatization." × 250

FIGURE 17.—Original tumor from which metastasis shown in figure 16 arose. × 50

FIGURE 18.—Narrow cords of duct cells and "stromatized" basal myoepithelial cells. × 50

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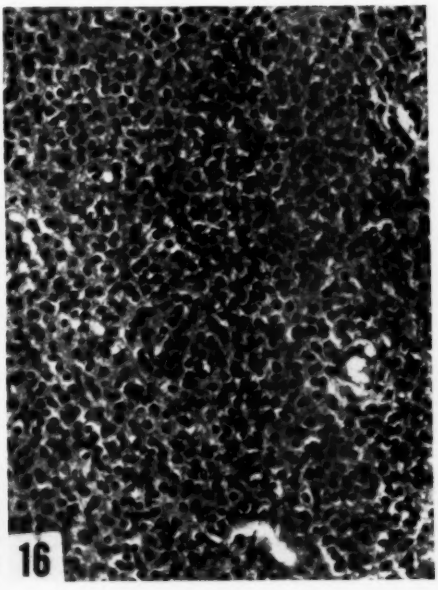
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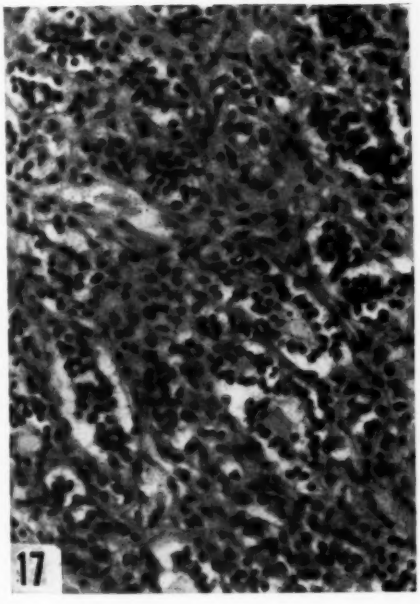
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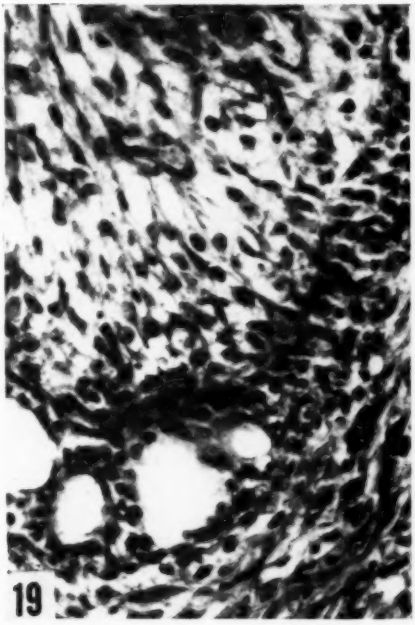
PLATE 33

FIGURE 19.—Area outlined in figure 18, showing myoepithelial cells that appear to have streamed from the duct tissuse into the stroma. × 250

FIGURE 20.—Whorls of myoepithelial cells that appear to have undergone transformation into stroma. Very few glandular structures and only a few residual duct spaces remain. × 50

FIGURE 21.—Another form of mixed tumor of the breast with large numbers of bizarre giant cells. × 35

FIGURE 22.—A reticulated pattern apparently the result of "stromatization" of myoepithelial tissue from adenocarcinoma of breast. × 200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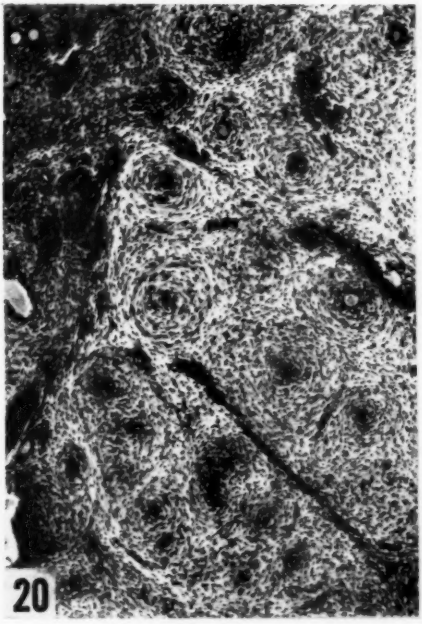
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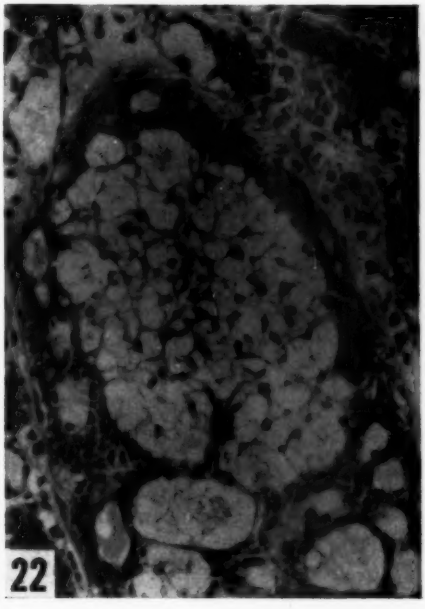
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Effect of Croton Oil on Induction of Tumors by 1,2-Benzanthracene, Desoxycholic Acid, or Low Doses of 20-Methylcholanthrene in Mice [1, 2]

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Papillomas of the skin have been induced repeatedly in mice that were painted once with a hydrocarbon carcinogen and then continuously with croton oil (*1–5, 8*). Tumors were not obtained, however, with a single dose of carcinogen or with croton oil alone. From these and related experiments, Berenblum and Shubik (*2*) concluded that painting with carcinogen was responsible for initiating the neoplastic change. During this phase, "latent tumor cells" were established that could be acted upon subsequently by a promoter such as croton oil, causing the altered cells to develop into visible tumors. The majority of growths so induced were benign. Skin carcinomas have been induced in greater number in other experiments in which mice were painted with a large dose of 20-methylcholanthrene (*5*) or with repeated doses of 3,4-benzpyrene, 20-methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene and croton oil (*6–8*). Smith (*9*) transplanted fetal skin and 1,2,5,6-dibenzanthracene in olive oil, with or without Scharlach R, to the hind limb of mice and found skin tumors at some of the injection sites. When croton oil was added to the inoculum, the incidence of tumors increased and the latent period decreased. In a recent study (*10*), in which mice were injected once intramuscularly with methylcholanthrene and croton oil or methylcholanthrene alone, the incidence of tumors was greater and the latent period shorter in those animals which received both carcinogen and croton oil. Sarcomas were observed in both groups. In experiments in which methylcholanthrene or benzpyrene was administered once percutaneously, the amount of carcinogen was low enough so that few or no tumors arose unless croton oil was applied repeatedly (*11–13*). One purpose of the present experiment was to determine whether croton oil injected once intramuscularly with progressively lower dilutions of methylcholanthrene would continue to influence the development of tumors even when few or no tumors were induced with the carcinogen alone.

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The carcinogenic activity of 1,2-benzanthracene, the parent compound of such active carcinogens as 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene, and 3,4-benzpyrene, has been investigated extensively in mice and rats. These compounds were found in most cases to produce few, if any, tumors (*14–16*). Steiner and Falk (*17*), however, injected 1,2-benzanthracene subcutaneously into mice and obtained sarcomas in 18 percent of their animals. The fact that 1,2-benzanthracene has been observed to be a weak carcinogen by most investigators (*14–16*) suggests this could be due to a lack either of initiating power or of promoting power, or both. Berenblum (*6*) painted the skin of mice repeatedly with this hydrocarbon and obtained no tumors. Addition of croton resin, the active principle of croton oil, to the benzanthracene solution also produced no tumors. Klein (*13*) painted the skin of mice once with methylcholanthrene in an attempt to produce "latent tumor cells," and then applied 1,2-benzanthracene in olive oil repeatedly at the same site. He observed that 1,2-benzanthracene induced no skin tumors under these conditions. In other experiments, Klein (*10*) found that in mice croton oil enhanced the capacity of methylcholanthrene to produce sarcomas when both agents were injected intramuscularly. It was therefore decided to test the action of 1,2-benzanthracene with croton oil following similar injection.

The chemical relationship of desoxycholic acid to the carcinogenic hydrocarbons is well known. Wieland and Dane (*18*) were able to synthesize methylcholanthrene directly from desoxycholic acid. Furthermore, methylcholanthrene has been prepared in the laboratory from cholic acid, another of the naturally occurring bile acids (*19*). The possibility that desoxycholic acid might be converted into a carcinogen *in vivo* led to its investigation in a number of laboratories. While some investigators found this compound to be devoid of carcinogenic activity, others reported induction of tumors with desoxycholic acid (*14*). Since desoxycholic acid appears to possess little or no carcinogenic activity, could this be the result of an inability to promote tumor development after the neoplastic change has been initiated? Inasmuch as croton oil was shown to be an effective promoter in skin tumorigenesis and appeared also to enhance the development of fibrosarcomas with methylcholanthrene, it was decided to investigate the effect of croton oil combined with desoxycholic acid in the mouse.

MATERIALS AND METHODS

Solutions of 20-methylcholanthrene,[5] croton oil,[6] and olive oil (U. S. P. grade) were prepared as follows: A stock solution of methylcholanthrene in olive oil containing 0.5 mg. of solute per ml. of solvent was refrigerated for 24 hours and then stored in the dark at room temperature. Lower dilutions, containing 0.05, 0.02, and 0.01 mg. of methylcholanthrene per ml., were prepared from aliquots of this stock solution by the addition of olive

[5] Obtained from Edcan Laboratories, South Norwalk, Conn. M. P., 179–180° C.
[6] Obtained from Fisher Scientific Company, Eimer and Amend, New York, N. Y.

oil. Each of the three dilutions was refrigerated for 1 day and brought to room temperature prior to use. Three olive-oil solutions containing 1 percent croton oil and 0.05, 0.02, or 0.01 mg. of methylcholanthrene per ml. were also required. These were prepared from half the volume of each corresponding methylcholanthrene-olive oil dilution. All solutions were refrigerated and stored as above.

Solutions of 1,2-benzanthracene[7] in olive oil and desoxycholic acid[8] in olive oil were each prepared so as to contain 4 mg. of solute per ml. of solvent. Six other solutions were prepared by the addition of croton oil in a concentration of 0.1, 1.0 and 3.0 percent to 3 aliquots of the benzanthracene-olive oil and desoxycholic acid-olive oil solutions. A 5.0 percent croton oil solution was similarly prepared, but using only benzanthracene.

Young adult male albino mice were used in the methylcholanthrene and benzanthracene series. These were derived from a colony, originally strain A, in which the agouti gene was discovered, indicating a possible outcrossing with another albino strain, probably C (BALB/c). In the desoxycholic acid series, young adult male strain C3H mice were employed. All mice were kept in plastic cages and had access to an unlimited supply of Purina laboratory chow pellets and tap water.

Each mouse was injected once intramuscularly in the right hind limb with one of the solutions described above. A ¼ ml. tuberculin syringe fitted with an 18-gauge, short-beveled hypodermic needle was employed for all injections. Each inoculum consisted of 0.04 ml. of test solution.

Excised tissues including tumors were fixed in Tellyesniczky's fluid, sectioned at 8 micra, and stained with hematoxylin and eosin. Diagnoses were established following microscopic examination.[9] Latent period was defined as the time in days for a tumor to become palpable.

EXPERIMENTAL PROCEDURES AND RESULTS

The mice that were injected with 20-methylcholanthrene (series A) were divided into seven groups of 40 each according to the concentration of carcinogen and the presence or absence of croton oil (table 1).

A1—0.05 mg. of methylcholanthrene per ml. of olive oil.

A2—0.05 mg. of methylcholanthrene per ml. of a 1 percent solution of croton oil in olive oil.

A3—0.02 mg. of methylcholanthrene per ml. of olive oil.

A4—0.02 mg. of methylcholanthrene per ml. of a 1 percent solution of croton oil in olive oil.

A5—0.01 mg. of methylcholanthrene per ml. of olive oil.

A6—0.01 mg. of methylcholanthrene per ml. of a 1 percent solution of croton oil in olive oil.

A7—1 percent solution of croton oil in olive oil.

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[7] Obtained from Eastman Kodak Company, Rochester, N. Y. M. P., 159–160° C.

[8] Obtained from Dr. C. D. Larsen, National Cancer Institute, Bethesda, Md. This compound, which was originally a commercial product, had been subjected to extensive purification. M. P., 174° C.

[9] The author wishes to thank Dr. Thelma B. Dunn of the Laboratory of Pathology of the National Cancer Institute for her assistance.

TABLE 1.—*Intramuscular injection of 20-methylcholanthrene, 1,2-benzanthracene, desoxycholic acid, and croton oil in male mice. Albino mice related to strain A were used in series A and B, and strain C3H mice in series C. Each mouse received 0.04 ml. of test solution.*

Series	Treatment (*concentration and solution of agent*)	Mice in experiment at start	Results					
			Effective total‖	Mice with tumors	Tumor incidence‖ (*percent*)	Mean latent period (*days*)	Average observation period, all mice (*days*)	Average age at death, all mice (*days*)
A1	0.05 mg. MCA* per ml. olive oil	40	35	5	14.3	183	350	440
A2	0.05 mg. MCA per ml. of 1 percent solution of croton oil in olive oil	40	36	3	8.3	185	344	454
A3	0.02 mg. MCA per ml. olive oil	40	36	1	2.8	254	362	450
A4	0.02 mg. MCA per ml. of 1 percent solution of croton oil in olive oil	40	35	1	2.9	272	355	460
A5	0.01 mg. MCA per ml. olive oil	40	33	0	....	....	378	464
A6	0.01 mg. MCA per ml. 1 percent solution of croton oil in olive oil	40	37	0	....	....	391	462
A7	1 percent croton oil in olive oil	40	35	0	....	....	382	455
B1	4.0 mg. BA† per ml. olive oil	35	32	0	....	....	376	456
B2	4.0 mg. BA per ml. 0.1 percent solution of croton oil in olive oil	35	30	0	....	....	368	447
B3	4.0 mg. BA per ml. 1.0 percent solution of croton oil in olive oil	35	24	3	12.5	377	381	466
B4	4.0 mg. BA per ml. 3.0 percent solution of croton oil in olive oil	35	26	1	3.8	375	384	458
B5	4.0 mg. BA per ml. 5.0 percent solution of croton oil in olive oil	35	30	0	....	....	367	442
C1	4.0 mg. DA‡ per ml. olive oil	40	....	0	....	....	355	439
C2	4.0 mg. DA per ml. 0.1 percent solution of croton oil in olive oil	40	....	0	....	....	342	442
C3	4.0 mg. DA per ml. 1.0 percent solution of croton oil in olive oil	40	....	0	....	....	332	423
C4	4.0 mg. DA per ml. 3.0 percent solution of croton oil in olive oil	40	....	0	....	....	353	455

*MCA—20-methylcholanthrene.
†BA—1,2-benzanthracene.
‡DA—desoxycholic acid.
§Mice alive at time first tumor was recorded in respective series.
‖Percent tumor incidence determined from effective totals.

The mice that were injected with 1,2-benzanthracene (series B) were divided into five groups of 35 mice each according to the concentration of croton oil. Each solution for series B contained 4.0 mg. of benzanthracene per ml. of olive oil. In addition, the solutions for series B2 to B5 inclusive contained croton oil in the following amounts: B2—0.1 percent; B3—1.0 percent; B4—3.0 percent; and B5—5.0 percent.

The mice that were injected with desoxycholic acid (series C) were divided into four groups of 40 mice each according to the concentration of croton oil. Each solution for series C contained 4.0 mg. of desoxycholic acid per ml. of olive oil. In addition, the solutions in series C2 to C4 inclusive contained croton oil in the following amounts: C2—0.1 percent; C3—1.0 percent; and C4—3.0 percent.

Ten tumors, all fibrosarcomas, were induced in the mice of the first four groups in series A (A1 to A4 inclusive) (table 1). In these, methylcholanthrene was administered in concentrations of 0.05 or 0.02 mg. per ml. of solvent. The solutions in series A2 and A4 contained, in addition, 1.0 percent croton oil. Of the ten tumors, five were observed in series A1, three in A2, and one each in A3 and A4; the tumor incidences of these series were 14.3, 8.3, 2.8, and 2.9 percent, respectively. No tumors were observed in any of the 105 mice of series A5, A6, and A7. With the injection of a smaller concentration of methylcholanthrene, the tumor incidence decreased while the latent period increased. Thus, when the concentration of carcinogen was 0.05 mg. per ml., the mean latent period was 183 days in series A1 and 185 days in series A2. When the carcinogenic solution contained only 0.02 mg. of methylcholanthrene per ml. of solvent, the mean latent period rose to 254 days in series A3 and to 272 days in series A4. The average period of observation for the mice of series A ranged from 344 days in series A2 to 391 days in series A6.

No tumors were found in the mice that received 1,2-benzanthracene in olive oil (series B1). Nor were any discovered when benzanthracene and 0.1 or 5.0 percent croton oil in olive oil was injected (series B2 and B5). A total of four tumors was found, however, in series B3 and B4 where the concentration of croton oil was 1.0 percent and 3.0 percent, respectively. Three of these neoplasms were hemangio-endotheliomas, and one a fibrosarcoma. The two hemangio-endotheliomas in series B3 were first palpable at an average latent period of 383 days, and the one in series B4 at 375 days—while the latent period for the fibrosarcoma in series B3 was 366 days.

In series C, in which 160 strain C3H mice were given injections of desoxycholic acid in olive oil or desoxycholic acid and 0.1, 1.0, or 3.0 percent croton oil in olive oil, none of the animals bore tumors after 332 days (series C3) to 355 days (series C1).

DISCUSSION

In a previous investigation (*10*), albino mice from the colony employed in the present experiments were injected once intramuscularly with 0.024 mg. or 0.004 mg. of methylcholanthrene in olive oil or in a 1 percent solution of croton oil in olive oil. While fibrosarcomas were induced in all four of

the series, the tumor incidence was greater and latent period shorter in those mice that received both carcinogen and croton oil. This suggested that croton oil, which promotes the development of skin tumors in mice, also enhanced the development of intramuscular sarcomas. In investigations on the initiating and promoting phase in tumorigenesis in the skin, mice were painted once with a low enough dose of carcinogen so that no tumors were induced unless this was followed by repeated applications of croton oil (*11–13*). In view of this, the concentration of methylcholanthrene was decreased in the present experiments to a point where few or no intramuscular neoplasms were found when the carcinogen alone was injected. Under these conditions, the incorporation of croton oil in the inoculum did not affect tumor incidence or latent period (table 1).

Why croton oil was effective when given with a comparatively large dose of methylcholanthrene and not with a smaller one is not known, but certainly deserves further study. The following considerations, however, may contribute to an understanding of this problem.

In experiments on induction of tumors in the skin involving one painting with a carcinogen, croton oil was shown to be an effective promoter when a carcinogen such as 9,10-dimethyl-1,2-benzanthracene was applied at a concentration of 0.06 percent (*4*). The actual dose was not given in the latter report. The carcinogen was applied with a glass rod. Assuming that each mouse received approximately 0.1 ml. of solution, this would be equivalent to 0.06 mg. of the carcinogen. In experiments where croton oil enhanced the activity of methylcholanthrene following intramuscular injection (*10*) each mouse received a total of 0.024 mg. or 0.004 mg. of the carcinogen. On the other hand, the total dose in the present experiments was decreased to 0.002 mg., 0.0008 mg., or 0.0004 mg. It is possible that a comparable decrease in the amount of carcinogen applied to the mouse's skin prior to the start of painting with croton oil would result, in like fashion, in a disappearance of the promoting effect observed at higher concentrations of the same carcinogen.

In the induction of skin tumors in mice, the nature of the resultant neoplasms appears to depend, in part, on the amount of carcinogen applied. Thus, with one low dose of methylcholanthrene, benzpyrene, or 9,10-dimethyl-1,2-benzanthracene followed by repeated paintings with croton oil, the tumors developed were predominantly benign papillomas (*5, 8, 12, 13*), whereas with a larger dose (*5*), or with repeated paintings of carcinogen (*6–8*), a greater number of malignant tumors were produced. All of the tumors induced intramuscularly in the present experiments with methylcholanthrene were examined microscopically and diagnosed as fibrosarcomas. These tumors enlarged rapidly, suggesting that they were malignant at the time of palpation. If, as has been observed for skin tumors in mice, a greater amount of carcinogen also is required to induce malignant tumors intramuscularly, one might expect few such tumors to arise following injection of so small a dose as 0.002 mg., 0.0008 mg., or 0.0004 mg. of methylcholanthrene. It will be observed that the incidence of fibrosarcomas obtained after injection of these three doses of carcinogen was 14.8, 2.8, and 0 percent, respectively (table 1). In the induction of skin tumors in mice, croton oil is not effective unless a carcinogen has first initiated the neoplastic change. Also, it would appear that croton oil is able to promote the development of malignant as well as benign tumors of the skin (*6, 7*). It may be that croton oil behaves similarly in the induction of fibrosarcomas so that if insufficient carcinogen were injected to initiate the malignant change, the addition of croton oil would be without effect. The failure of croton oil to influence tumorigenesis in the present experiments might be explainable on this basis.

Based on the experiments on induction of skin tumors, one of the requirements for a cocarcinogen involves direct action upon a tissue (*20*). Thus, croton oil may promote the development of skin tumors in the mouse even when applied 43 weeks following

a single painting with a carcinogen when the latter is no longer presumed to be present (*21*). It is possible that the increased tumor incidence and decreased latent period observed following intramuscular injection of croton oil and a large dose of methylcholanthrene (*10*) may have been the result of indirect rather than direct action upon the surrounding tissues. Thus, the effectiveness of the methylcholanthrene may have been favored by a change in the degree of solubility, the rate of metabolism, or the rate of removal of the carcinogen. Under any of these conditions, no cocarcinogenic activity could be ascribed to croton oil.

No tumors were observed in mice that had been injected with 1,2-benzanthracene in olive oil. This agrees with the results of most investigators who also found this hydrocarbon lacking in carcinogenic potency (*14*). However, a total of three hemangio-endotheliomas and one fibrosarcoma was observed when croton oil in a concentration of 1.0 or 3.0 percent was added to the benzanthracene solution (table 1). Spontaneous hemangio-endotheliomas [19] and fibrosarcomas occur infrequently in strain A mice.

Thus, while the incidence of such tumors in untreated mice of this strain is unknown, it would appear that hemangio-endothelioma and probably fibrosarcoma as well were induced. Fibrosarcomas are induced readily in strain A mice, and hemangio-endotheliomas have been observed repeatedly following administration of hydrocarbons known to be carcinogens (*22–27*). The significance of croton oil in enhancing the tumorigenic potency of 1,2-benzanthracene is not apparent from these experiments. While croton oil injected intramuscularly may have acted directly on the surrounding tissues, this may have occurred indirectly by an increase in solubility or a change in the rate at which 1,2-benzanthracene was metabolized. Despite injection of the hydrocarbon in high concentration (4 mg. per ml.) in a susceptible strain and the addition of croton oil to some of the benzanthracene solutions, only 4 of 142 mice bore tumors, an incidence of 2.8 percent in series B (table 1). However, in series B3, the mice of which received 1,2-benzanthracene and 1.0 percent croton oil in olive oil, the incidence of tumors was 12.5 percent. Hence, while 1,2-benzanthracene in itself may possess little or no carcinogenic activity under certain experimental conditions, its limited activity appears to be enhanced.

Although desoxycholic acid was reported in some instances to be slightly carcinogenic in mice and rats, it usually failed to produce tumors in these species (*14*). While desoxycholic acid may be converted to methylcholanthrene (*16*) in the laboratory, it would appear from the results obtained in the present experiments that this does not occur *in vivo* in the mouse following intramuscular injection. The failure to induce tumors in C3H mice, a strain which is highly susceptible when injected with the hydrocarbon carcinogens (*28*), indicates once more that desoxycholic acid lacks tumorigenic activity. Also, this inactivity does not appear to be influenced by the addition of croton oil in concentrations of 0.1, 1.0, or 3.0 percent.

SUMMARY

Albino mice related to strain A were injected intramuscularly with *a*) 0.002 mg., 0.0008 mg., or 0.0004 mg. of methylcholanthrene in olive oil or *b*) the same solutions containing, in addition, 1 percent croton oil. Tumors were induced with the 0.002-mg. and 0.0008-mg. doses, but not with the 0.0004-mg. dose. The addition of croton oil had no observable influence either on tumor incidence or latent period. These observations are compared with those from an earlier experiment in which larger

[19] Personal communication from Dr. C. C. Little and Dr. W. E. Heston.

amounts of methylcholanthrene were employed and in which sarcogenesis was enhanced with croton oil.

Mice from the same colony were injected intramuscularly with 1,2-benzanthracene in olive oil or 1,2-benzanthracene and 0.1, 1.0, 3.0, or 5.0 percent croton oil in olive oil. Tumors were observed only in those mice which received the hydrocarbon and 1.0 or 3.0 percent croton oil. It is not known whether croton oil exerted a direct or an indirect action in enhancing tumorigenesis.

Strain C3H mice were injected intramuscularly with desoxycholic acid in olive oil, or desoxycholic acid and 0.1, 1.0, or 3.0 percent croton oil in olive oil. None of the mice bore tumors after 332 to 355 days.

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Effect of Injected Lyophilized Tumor and Trypan Blue on Host Resistance to Tumor Grafts [1,2]

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It has been shown that vital staining of the host with dyes like trypan blue and congo red results in increased growth of transplanted tumors in the mouse and the rabbit when such grafts would have otherwise usually regressed (*1–4*). These effects are presumably due primarily to impairment of the reticulo-endothelial system, with a resultant loss of natural resistance to a given tumor graft.

It has also been demonstrated that injection of lyophilized mouse tissues, fresh tissue homogenates, and antiserums to these tissues prior to implantation of a tumor cause a loss of resistance of inbred mice to tumor homoiografts (*5–8*). Very small doses evoke an increased resistance to such grafts (*9*). The present paper reports experiments designed to elucidate the mode of action of the lyophilized tissues, and to determine whether this action has a basis common to lyophilized tissues and vital dyes.

MATERIALS AND METHODS

The test tumor used was the transplantable tumor 15091a, an anaplastic carcinoma of the mammary glands which is indigenous to the inbred A strain of mice. It grows rapidly in 100 percent of strain A animals of both sexes. Most of the hosts die within 3 to 5 weeks after subcutaneous inoculation, but an occasional mouse may survive as long as 8 weeks. The test hosts were C57BL/6Ks strain mice. Tumor 15091a grows progressively in about 1 of 300 of these mice. The mice ranged in age from 2 to 3 months at the start of the experiments, and were about equally divided as to sex. All mice appeared healthy and vigorous, though they were not tested for latent enteric or respiratory infection. All the mice were weighed at the beginning of the experiment, and periodically for 2 months thereafter, up to the time of grafting of the tumor.

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The materials injected were trypan blue and lyophilized tumor 15091a. The tumor was derived from subcutaneous implants grown in mature strain A/Ks mice which had been inoculated 11 to 12 days prior to harvesting of the tumor. The tumors were removed under aseptic conditions and kept at the temperature of melting ice until they were frozen for lyophilization. They were then frozen rapidly over dry ice and lyophilized. After drying, the tissue was powdered and sealed under vacuum in ampoules. For purposes of injection, the lyophilized tumor was ground in sterile 0.85 percent sodium chloride solution in glass homogenizers. The resulting suspension was kept cold for injection. Injections were made intraperitoneally in 0.5 ml. amounts.

The trypan blue was made up in sterile solution in 0.85 percent sodium chloride, in a concentration of 5 mg. per ml. (0.5 percent). Injections were given intraperitoneally in 0.2 ml. amounts.

The protocol of injections is given in table 1. The mice were divided into four categories, in terms of the materials injected: 1) trypan blue alone, 2) lyophilized tumor alone, 3) trypan blue and lyophilized tumor, and 4) controls (nothing injected). The time relationships of injections for the groups of mice receiving both trypan blue and lyophilized tumor are shown in text-figure 1. All the mice received inoculations of live tumor after the courses of injections were stopped. The time of inoculation was 81 days after the start of the trypan blue injections, and corresponded to 1 week after the last injection of lyophilized tumor.

TABLE 1.—*Protocol of injections with trypan blue and lyophilized tumor 15091a**

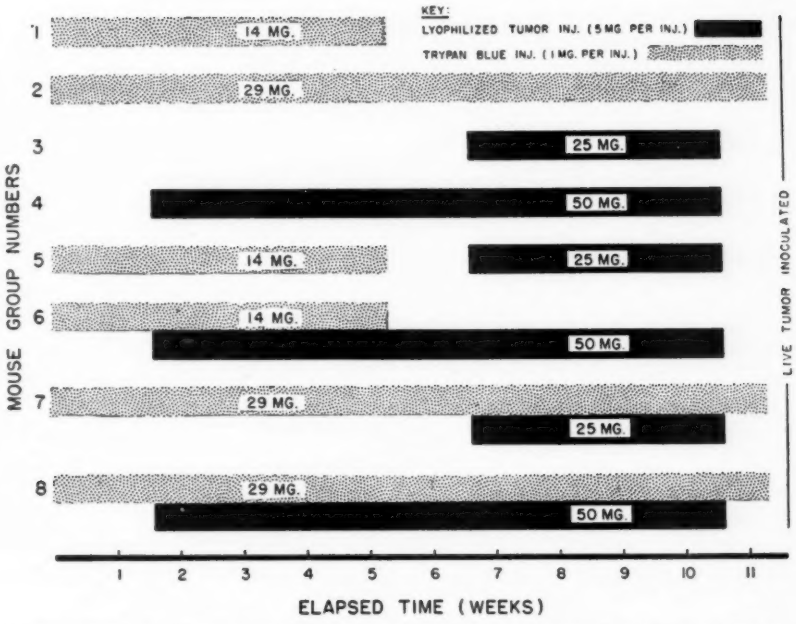
Experimental group No.	Substance injected	Total amount per mouse (*mg., dry wt.*)
1	Trypan blue	†14
2	Trypan blue	‡29
3	Lyophilized tumor	25
4	Lyophilized tumor	50
5	Trypan blue	†14
	Lyophilized tumor	25
6	Trypan blue	†14
	Lyophilized tumor	50
7	Trypan blue	‡29
	Lyophilized tumor	25
8	Trypan blue	‡29
	Lyophilized tumor	50
9	Nothing	. . . . .

*All injections given intraperitoneally; all animals inoculated with live tumor on the same day (corresponding to 1 week after the last injection of lyophilized tumor). Vehicle for both the dye and the lyophilized tissue was sterile 0.85 percent sodium chloride. Dye injections given approximately every third day in 0.2 ml. amount per injection, containing 1 mg. of dye; lyophilized tumor injections given weekly in 0.5 ml. amount per injection, containing 5 mg. of tissue.

† Total of 14 injections, which were stopped 6 weeks before live tumor inoculation.

‡ Total of 29 injections, which were stopped 2 days before live tumor inoculation.

A single inoculation of bits of minced live tumor was made subcutaneously by trocar, under aseptic conditions, into the right suprascapular region. The course of growth of the grafts was followed by palpation,



TEXT-FIGURE 1.—Time relationships for the courses of injections of trypan blue and lyophilized tumor.

until the animals either died or were recorded as negative for a consecutive period of 3 to 4 months. Autopsies were performed on the mice, whether they died with tumors or were classified as negative.

RESULTS

No overt signs of toxicity were observed throughout the injection period. All animals gained weight in normal manner. Retention of the dye with generalized distribution throughout the body was found as long as 5 months after the injections had ceased. Coloration to a marked degree was found in the subcutaneous tissue, kidney, liver, spleen, adrenal gland, testis, uterus, and ovary. The muscles and thymus were less markedly blue. The lungs had a very faint trace of color. No metastases were found in animals dying with tumors.

Table 2 presents the data on the number of animals dying with tumors. The difference between the group receiving trypan blue alone and the controls is not significant. It is clear that the injection of lyophilized tumor abrogated the resistance of the hosts to the tumor grafts. There appears to be a significant difference between the groups receiving either lyophilized tissue plus trypan blue or lyophilized tissue alone ($\chi^2=5.00$, $P>0.02$), the former group having a larger number of animals with "takes" (98 percent as against 83 percent).

TABLE 2.—*Number of C57BL/6Ks mice dying with tumors*

Experiment group No.	Substance injected		Mice dying* † with tumors
	Substance	Amount (*mg.*)	
1	Trypan blue	14	0/10
2	Trypan blue	29	0/10
3	Lyophilized tumor	25	17/20
4	Lyophilized tumor	50	16/20
5	Trypan blue	14	11/11
	Lyophilized tumor	25	
6	Trypan blue	14	9/10
	Lyophilized tumor	50	
7	Trypan blue	29	9/9
	Lyophilized tumor	25	
8	Trypan blue	29	10/10
	Lyophilized tumor	50	
9	Nothing	.....	0/21

*The numerator is the number of animals dying with tumors; the denominator is the total number of animals in the group.

†Comparison of the totals for groups 3 and 4 (33/40) with that for groups 5, 6, 7, and 8 (39/40) gives a χ^2 of 5.00, $P > 0.02$.

DISCUSSION

Under the conditions of our experiments, the injection of trypan blue alone did not abrogate the resistance of the hosts to the tumor grafts, whereas injection of lyophilized tumor did so. There is an indication of a synergistic effect between the dye and the lyophilized tumor.

Our failure to get positive results with the dye alone, in contrast to previous reports in the literature (*1-4*), may be due to the differences in experimental procedures. In our case, injections of the dye were discontinued prior to tumor inoculation, while other investigators continued administration of the dye after inoculation of the tumor. This point warrants further investigation.

Our results do not answer the question concerning the mode of action of the lyophilized tissue. The possible synergism between trypan blue and lyophilized tumor may, or may not, be due to a common action in the host.

SUMMARY

The comparative effects of injection of trypan blue and lyophilized tumor, prior to inoculation of the tumor implant, on the course of growth of tumor homoiografts in mice. Trypan blue alone had no effect, while the injection of lyophilized tumor alone abrogated the normal resistance of the host to the graft. There was an indication of a synergistic effect of the dye and lyophilized tumor, since the groups of mice receiving injections of both substances exhibited a higher number of tumor "takes" as compared with the groups injected with lyophilized tumor alone.

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Massive Tissue Cultures Prepared from Whole Chick Embryos Planted as a Cell Suspension on Glass Substrate [1]

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In previous papers (*1–4*), methods have been described by which cell suspensions were prepared from cultures of cell strains maintained *in vitro* for extended periods of time. Such cell suspensions were used to plant large numbers of replicate cultures of strain L mouse fibroblasts (*2, 4*) and to obtain massive cultures of strain L mouse fibroblasts and of chick-heart fibroblasts (*1, 3*). The cell strains in these early studies had been originally established *in vitro* by planting single tissue fragments either immediately under perforated cellophane (*3*) or first in plasma (*5*), with later transfer to cellophane (*6, 7*). Once luxuriant growth was obtained under perforated cellophane, it was possible to prepare cell suspensions with a population density sufficient to establish cultures on the glass floor of the culture flasks (*3, 4*).

The purpose of the present study was to determine whether massive tissue cultures could be established directly on glass substrate by using cell suspensions prepared from fresh embryonic tissue.

GENERAL MATERIALS AND METHODS

Nine-day chick embryos were chosen as the material for this study. The culture flasks used were of the T-60 type (*8*) which has a floor area of approximately 60 sq. cm. The cleaning and sterilization of glassware, preparation of culture medium, and fluid renewal procedures were essentially according to the methods described by Earle (*5, 9*). The stock culture medium consisted of 20 percent embryo extract, 40 percent horse serum, and 40 percent Earle's balanced saline. The embryo extract was prepared by 1:1 saline extraction of the pulp from 9-day chick embryos. The medium on all cultures was renewed three times weekly and the cultures were gassed with 5 percent CO_2 in air to adjust and maintain the *p*H of the fluid at approximately 7.3. At the first fluid renewal, 24 hours

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after planting, the old supernatant fluid with its free-floating cells was withdrawn and 6 ml. of fresh nutrient was added to each culture. During the following fluid renewals, the old supernatant fluid was withdrawn and increasing amounts of fresh nutrient fluid were added until the total volume of fluid on each culture was 8 ml. The cultures were maintained from then on by replacement of this amount of nutrient fluid at each fluid renewal.

The procedure for preparing the cell suspensions and planting the cultures was as follows: The embryos were washed in a suspending medium which consisted of either Earle's balanced saline or a mixture of Earle's saline and filtered horse serum, and then were passed through a circular piece of 24-mesh stainless steel gauze fitted in the bottom of a 50 ml. Luer syringe (*5*). The resulting pulp was received in a 100 ml. centrifuge tube and was thoroughly agitated in a given volume of suspending medium. The coarse suspension was then passed through a double-mesh sieving unit (*2*) to obtain a fine, relatively uniform suspension of single cells and cell aggregates. This sieving unit contained two separate platinum alloy sieves of 80 and 150 mesh respectively, fixed 10 mm. apart in the bottom of a modified glass separatory funnel. Five- or ten-ml. aliquots of the sieved suspension were used to plant the cultures on the glass floor of the T-60 flasks.

EXPERIMENTS AND RESULTS

Preliminary attempts to establish cultures directly from the concentrated tissue pulp that remained after routine preparation of embryo extract resulted in complete failure. Within 3 days most of the tissue was necrotic and very few living cells could be detected in the culture flasks. All cultures of four series, A, B, C, and D, initiated by the present sieving procedure, grew actively for extended periods of time. The cultures in all series except D were maintained for at least 100 days. One culture from each series was continued at least 200 days for further observation. The cultures of series D were maintained for 33 days.

Cultures Planted With Cells Suspended in Horse Serum–Saline Mixtures

In series A the culture flasks were inoculated with two different concentrations of cell suspension. The lighter inoculum was prepared from one whole embryo, and the denser inoculum from two whole embryos. In each case the cells were suspended in 40 ml. of 20 percent horse serum in saline. The cells, other than red blood cells, in aliquots of the light inoculum were enumerated using a hemocytometer, and the cell concentration was estimated to be approximately 1,000,000 cells per ml. of suspension. Four cultures were planted from each inoculum. Of these, 2 flasks received 5 ml. portions, while 2 flasks received 10 ml. portions.

In series B the cells from a single whole embryo were suspended in 50 ml. of 5 percent horse serum in saline. Six T-60 flasks were planted with 5 ml. portions of this cell suspension.

There appeared to be no marked difference in the growth of the cultures planted with cells suspended in the 5 percent or the 20 percent horse

serum–saline mixtures. Twenty-four hours after the cultures were planted, many small aggregates of cells and single cells were attached to, and evenly distributed over, the glass surface of the culture flasks.

Within 5 days, cell migration and proliferation were very active. The cells migrated out from most of the small aggregates, the spaces between the aggregates were rapidly filled in (fig. 1), and a thin continuous cell sheet was formed. In some areas the glass surface was incompletely covered by the cell sheet and small perforations occurred. In a few of the cultures small foci of necrotic tissue were present.

Within two weeks, cell growth was luxuriant and a thick continuous cell sheet covered the floor of the culture flask. However, in all cultures planted with a dense suspension, small sections of the cell sheet came loose from the surface of the glass and the whole sheet became ragged and torn in appearance. Also many of the cell aggregates became necrotic, instead of taking an active part in forming the cell sheet.

After one month the cell sheet was very thick and opaque, as shown in a culture from series B (fig. 2). The cells forming the sheet were closely packed and a few small foci of necrotic tissue were present. Retraction of the edges of the cell sheet and fenestration in the sheet occurred to some extent in all cultures. This was especially severe in all the cultures planted with dense suspensions. In cultures that were maintained for more than 6 months in the original culture flask, there was much retraction of the edges of the cell sheet, accompanied by diffuse necrosis. Cleared areas of the glass surface, which resulted from the retraction of the main cell sheet, were rapidly covered again by cell migration and proliferation.

Since it was not within the scope of this study to attempt a detailed classification of the cells, efforts in this direction were limited only to microscopic observation and photomicrography of living, unstained cultures. However, many different types of cells were observed, especially within the first five days or so of culture growth. The majority of the cells tended to become elongate and fusiform upon migration from the original cell aggregates. These cells included, among other types, multinucleated skeletal muscle cells, smooth muscle cells, and fibroblast-like cells. Some of the cells had a low refractive index and formed thin sheets with the mosaic pattern characteristic of epithelial cells. Others had extremely long filamentous processes extending in opposite directions from a central cell body, suggesting nerve cells. Many cells were rounded in shape and some were amoeboid-like, similar to leukocytes and macrophages.

As the cultures progressed in age the cells became closely packed together and the cell population appeared to be more homogeneous. However, it was still possible to distinguish various cell types. Long muscle fibers (fig. 3) contracted spasmodically as early as 2 weeks in culture and as late as 6 months. Cells suggestive of macrophages (fig. 4) covered areas of the glass surface exposed by perforations in the cell sheet. Filamentous elements (fig. 5) were observed, but it was not determined whether they were nerve fibers or not. In extensive areas of one of the cultures, the cells assumed an elaborate, organized architecture (figs. 6, 7)

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that consisted of rounded bodies surrounded by zones of radially oriented cells. The rounded bodies ranged in diameter from 25 μ to 150 μ, appeared to be granular, and exhibited structural homogeneity in the sense that no cellular detail could be detected. However, this appearance of an absence of cellular detail may have been due to the thickness of the rounded bodies.

In series C, the heads were detached from 5 embryos and the cells from the head and the neck region were suspended in 30 ml. of 5 percent horse serum in saline. Five ml. portions of this dense cell suspension were pipetted into each of four T-60 flasks. The culturing procedure after planting was the same as that described above, except for one culture which received 4 ml. of fresh embryo extract at each fluid renewal for the first week and from then on received 8 ml. of stock medium.

In this group of cultures many more sheets of epithelial-like cells were present. In the single culture that had been treated with embryo extract alone, the epithelial-like sheets were even more extensive (fig. 8). Many of the cells in these sheets contained brown pigment (fig. 9). The pigmented areas were easily visible to the naked eye by either transmitted or reflected light. Eventually, small pockets or droplets of colorless material, perhaps secretory in nature, developed in the pigmented areas (fig. 10), and this was followed by necrosis of the cells.

Cultures Planted with Cells Suspended in Earle's Saline

In series D, the cells from a single whole embryo were suspended in 50 ml. of Earle's saline, and 5 ml. portions of this suspension were used to plant 6 cultures. Twenty-four hours later, the old supernatant fluid with free-floating cells was withdrawn from the flasks and the cultures were treated with different nutrient media. At 24 hours, just before addition of the various nutrient media, there was much less evidence of cell migration and proliferation in these cultures than in the cultures planted with cells suspended in horse serum–saline mixtures.

In three cultures of this group that were then treated for 3 days with 40 percent embryo extract in Earle's saline, the cells were grouped together in small discrete clumps about 0.1 to 0.2 mm. in diameter, and the intervening areas were almost free of cells. By sharp contrast, in three cultures that were treated for the same period of time with 40 percent horse serum in Earle's saline, many cells had migrated from the small aggregates and had spread diffusely over the areas that previously were free of cells. Relatively few of the original cell aggregates remained and a thin continuous layer of cells was formed.

One of the cultures treated with embryo extract continued to receive extract treatment and the cells remained in excellent condition. In time they migrated radially out from the discrete clumps, and by 30 days had formed a thick continuous cell sheet. One of the cultures continued to receive horse-serum treatment and the cells were in poor condition. By 30 days much cellular debris was present, and the cell population appeared to be less dense than in any of the other cultures.

The cultures that were treated with stock culture medium after previous treatment with embryo-extract mixture showed a luxuriant migration of cells in 3 days, and within 21 days a thick continuous cell sheet was formed. Those cultures that were treated with stock medium after previous treatment with horse-serum mixture showed a similar migration pattern, but there was a growth lag of about one week and also more foci of necrotic cells.

DISCUSSION

An analysis of the failure of the preliminary attempts to establish cultures from the concentrated tissue pulp of the chicken embryos suggested the following possible causes: *a*) The refrigerated centrifugation of the tissue pulp at 2300 r.p.m. (1600 G) during the extraction procedure (*5*) may have had a detrimental effect on the cells; *b*) The many red blood corpuscles that were present could possibly have had a phototoxic effect (*10*); *c*) The amount of tissue was so great that its needs probably far exceeded the available nutrient (oxygen) or the surface substrate in the culture vessel (*11*); *d*) The tissue was in relatively large thick fragments, which would tend to become necrotic more readily than smaller and thinner fragments (*12*).

In order to take these points into account in the present study the following things were done: the centrifugation of the pulp was eliminated since it was not only unnecessary but probably harmful; each embryo was washed thoroughly to remove as many red blood cells as possible; the quantity of tissue pulp obtained from a single 9-day embryo was suspended in 30 to 50 ml. of medium to facilitate further sieving and to eventually attain an inoculum comparable to that found to be most effective for the growth of strain L cells (*11*); and finally, the coarse suspension was passed through the 80–150 double-mesh sieve to obtain a fine, relatively uniform suspension of single cells and cell aggregates.

Of the above factors, the cell concentration in the suspensions used for planting was considered to be of critical importance in the successful establishment of cultures. With cultures of strain L mouse cells in T-12 flasks, it has been shown (*11*) that use of inocula ranging in size from 100,000 to 3,000,000 cells per flask resulted in an increase in cell population, while use of an inoculum as dense as 5,500,000 cells per flask resulted in a marked decrease in cell population and much cell necrosis.

Obviously many cells were destroyed or injured when the tissue was passed through the coarse mesh in the Luer syringe. Also some of the tissue remained intact as relatively large fragments. However, sufficient numbers of living cells were obtained after the fine sieving so that an effective inoculum could be prepared to establish luxuriant cultures consistently.

All the cultures in this study, except series D, were successfully initiated using cells suspended in a horse serum–saline mixture. The results in series D indicated that cultures could also be established using cells suspended in balanced saline alone. The cells, however, were arranged more definitely as small clumps, and cell migration was limited. The growth

pattern in these cultures was markedly different when treated later with various culture media.

The probability that this cell-suspension method could be adapted to the preparation of cultures of other embryos, whole organs, tumors, or other soft tissues, warrants further investigation. The procedure is simple and a cell suspension can be prepared from a single chick embryo in less than 20 minutes. Since the cultures may be easily and rapidly prepared in large numbers, they may well be of particular significance for the cultivation of viruses and microorganisms, and also in the study of cell differentiation and tissue organization.

SUMMARY AND CONCLUSIONS

1) A method is described by which cell suspensions were easily and rapidly prepared from whole 9-day chick embryos by passing the embryos through specially arranged sieves of consecutively finer mesh.

2) These cell suspensions were used to establish massive cultures, 60 sq. cm. in area, of embryonic tissue directly on glass substrate in T-60 flasks. In less than 1 week a thin layer of actively proliferating cells covered the floor of the culture flasks, and within 2 weeks luxuriant cell sheets were formed. The size of the cell aggregate, the concentration of the cell suspension, and the quantity used for inoculation are considered to be of critical importance in the successful establishment of the cultures.

3) Many different types of cells, including fibroblast-like cells, macrophage-like cells, epithelial-like cells, and muscle fibers, were actively growing in these cultures. Some cells formed elaborate organoid structures. Spasmodic contraction of muscle fibers occurred as early as 2 weeks in culture and as late as 6 months. Different types of epithelia, nonpigmented and pigmented, were actively proliferating after 8 months of cultivation. The data suggest that by planting and culturing the cells in different media, some selective effect can be produced with regard to the types of cells which predominate in the cultures.

4) It appears that this cell-suspension method may be used for planting large cultures from other embryos, whole organs, tumors, and other soft tissue. Since such cultures may be easily and rapidly prepared in large numbers, they may well be of particular significance for the cultivation of viruses and microorganisms, and also in the study of cell differentiation and tissue organization.

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PLATE 34

FIGURE 1.—A living, unstained culture from series A photographed 5 days after inoculation with a 5 ml. aliquot of cell suspension prepared from a single 9-day chick embryo. Note the even distribution of the tissue adhering to the glass floor of the flask. The scale shown is for both figures 1 and 2 and is graduated in millimeters.

FIGURE 2.—A living unstained culture from series B at 47 days of age. Note that the thick continuous cell sheet covers almost the entire floor of the flask. The dark areas are holes through the main cell sheet.

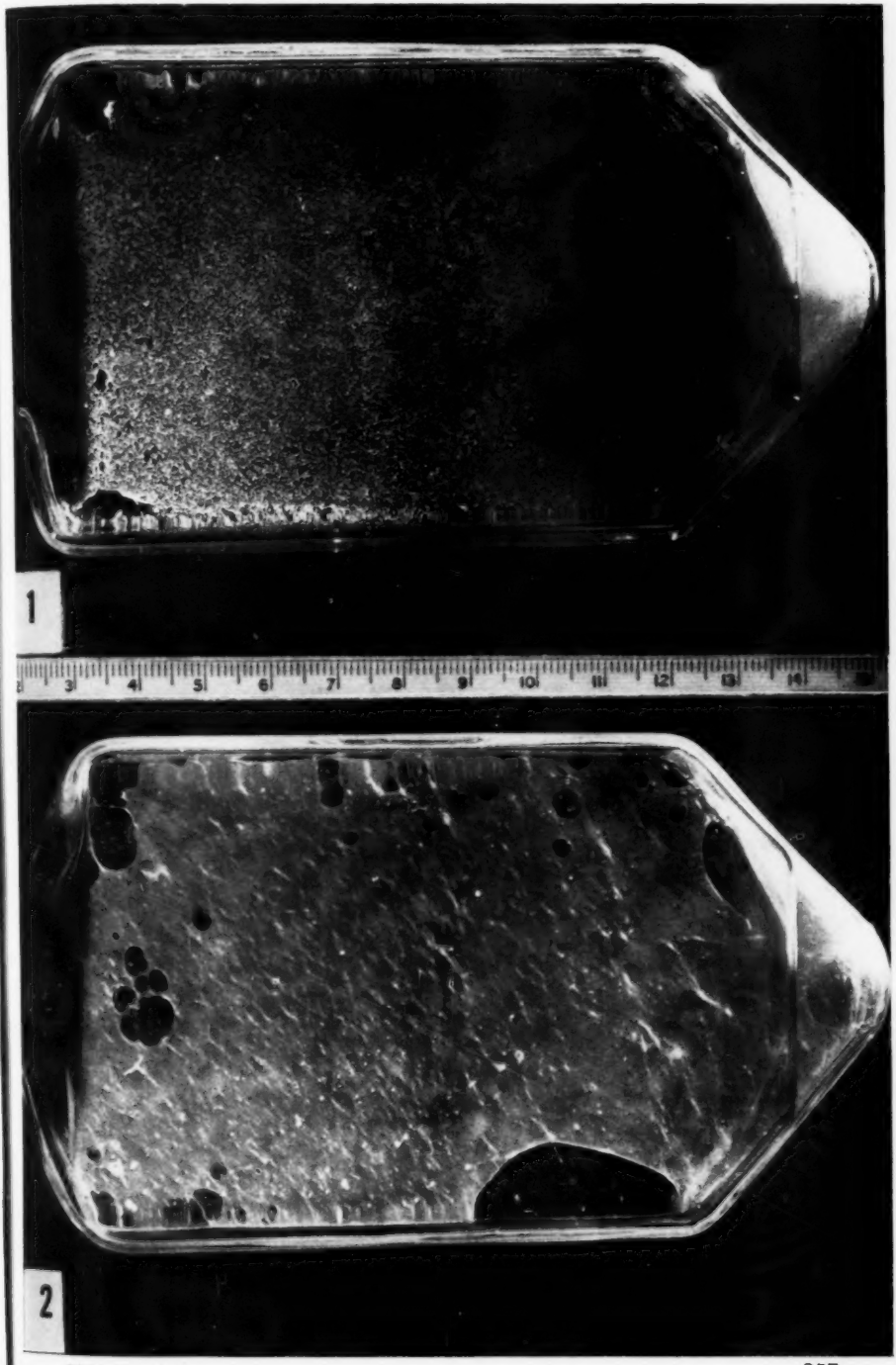

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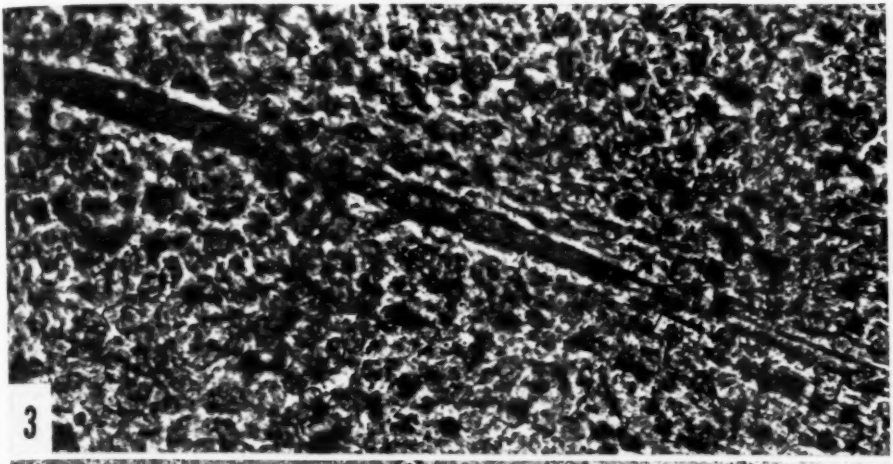
PLATE 35

FIGURE 3.—Photograph of a contractile muscle fiber embedded in the closely packed matrix of cells composing the cell sheet of the culture shown in figure 2. × 200

FIGURE 4.—Photograph of macrophage-like cells covering the glass surface in the perforated areas of the cell sheet of a 47-day-old culture from series B. × 200

FIGURE 5.—Photograph taken after 6 months of growth, showing filamentous elements present in a culture of series B. × 200



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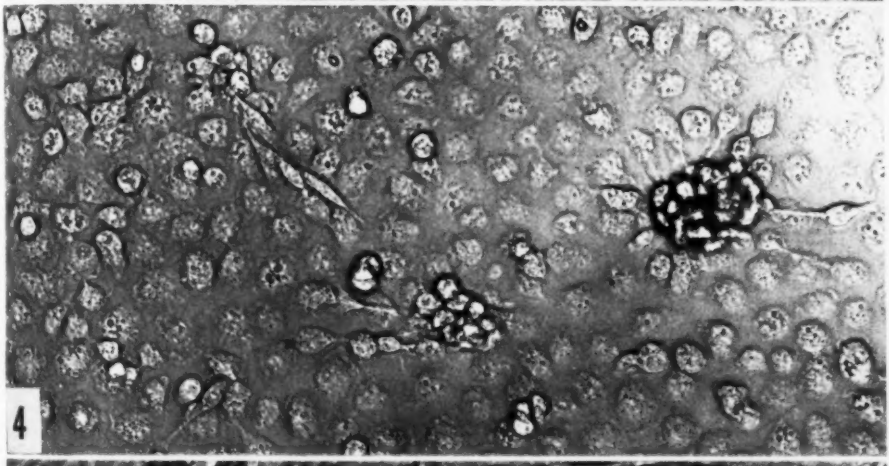

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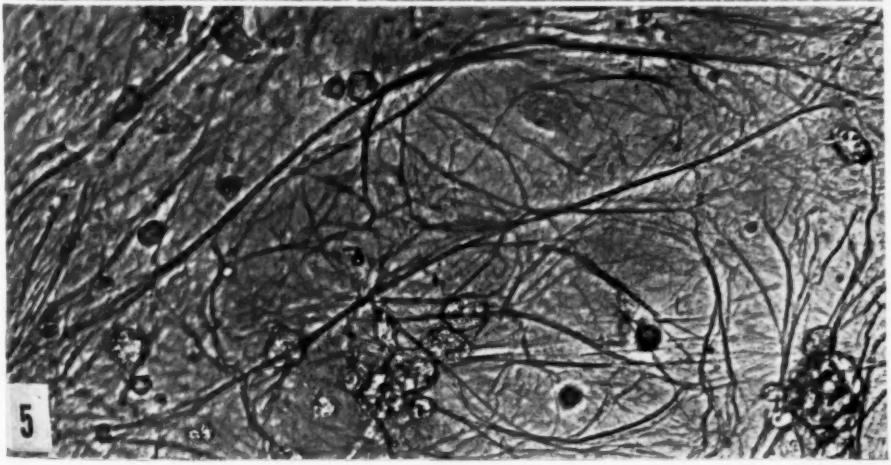

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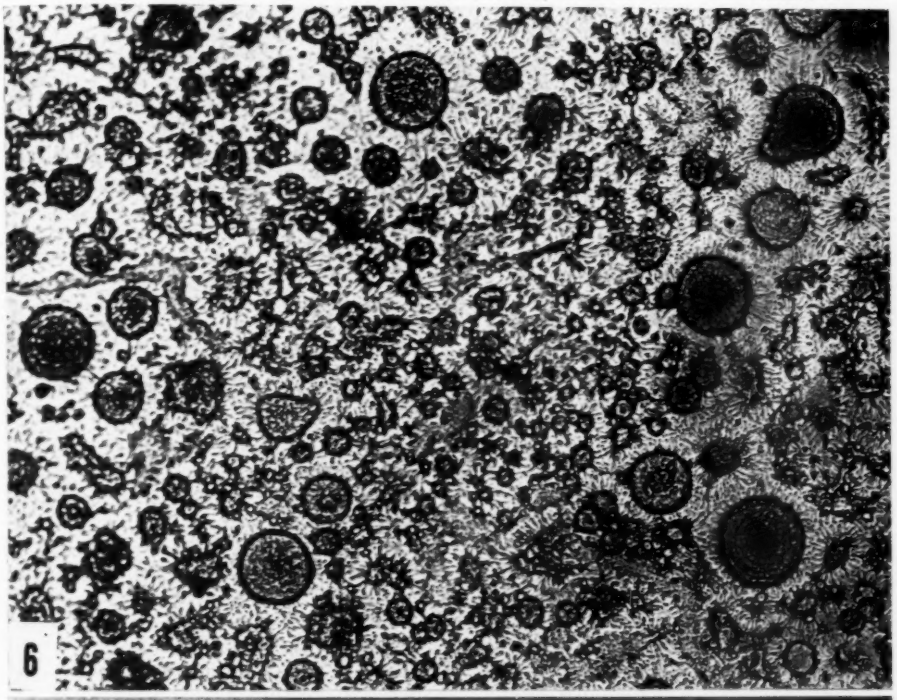
PLATE 36

FIGURE 6.—Photograph showing organoid structures in the cell sheet of a 5-month-old culture from series B. Note the concentric arrangement in the rounded bodies and the surrounding zones of radially oriented cells. × 80

FIGURE 7.—Photograph taken at higher magnification along the edge of an area containing the organoid structures. The zones of radiating cells are more clearly shown. × 200



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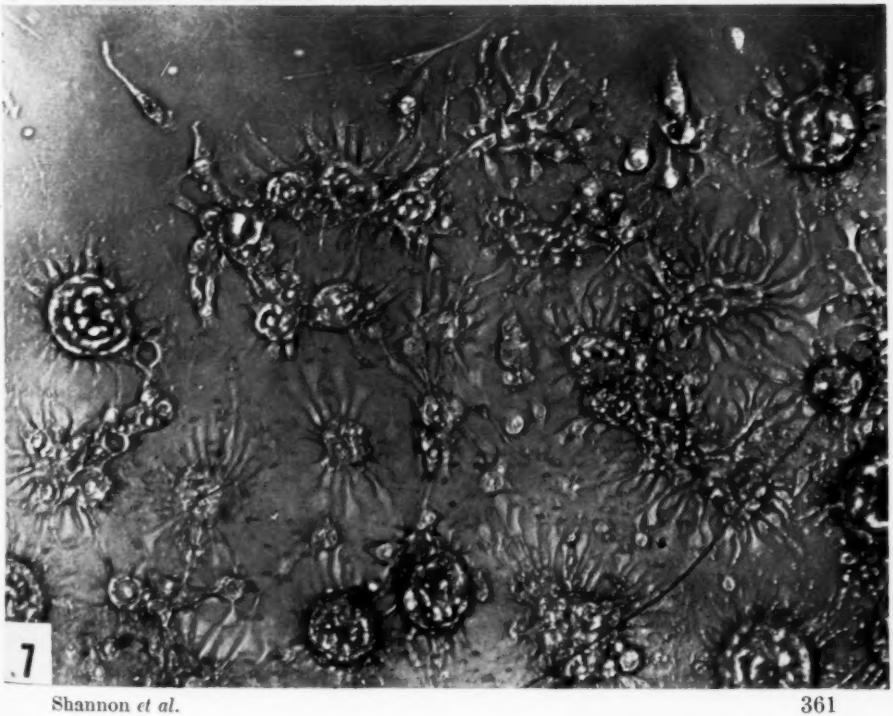

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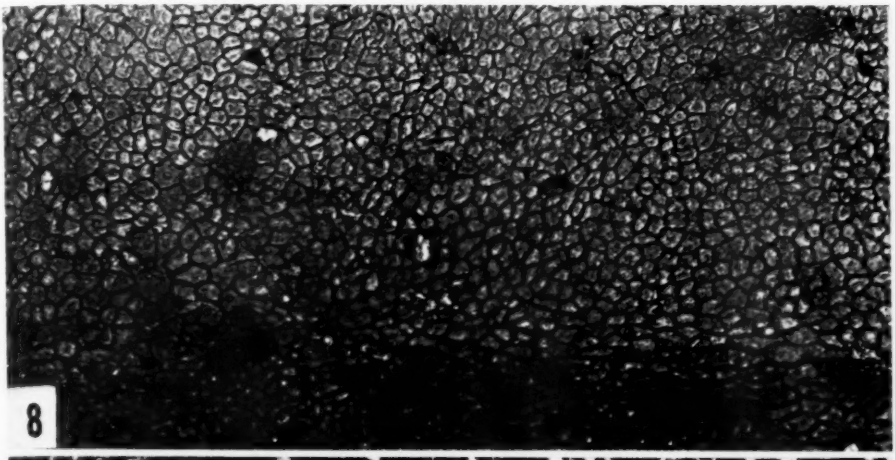
PLATE 37

FIGURE 8.—Photograph taken after 4 months of growth, showing part of one of the epithelial sheets representative of the cultures in series C. × 200

FIGURE 9.—Photograph also taken after 4 months of growth, showing part of a pigmented area in the cell sheet of the series C culture that received embryo-extract treatment. × 200

FIGURE 10.—Photograph showing the small pockets or droplets of colorless material which developed in the pigmented areas. × 200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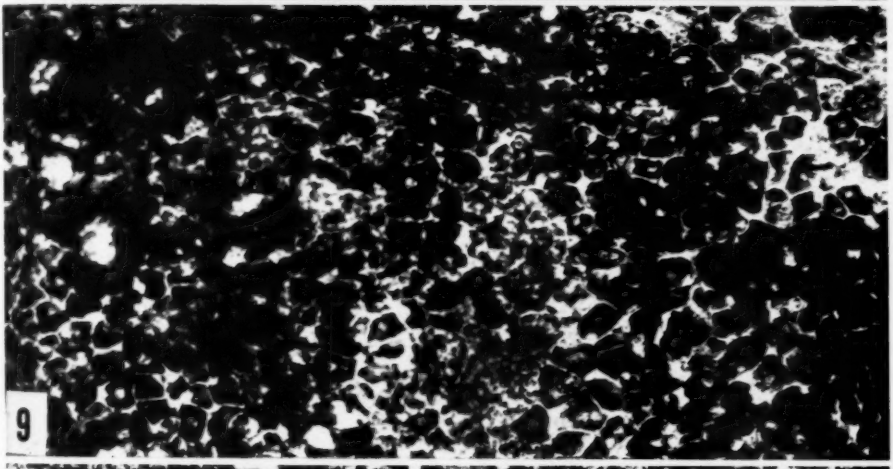
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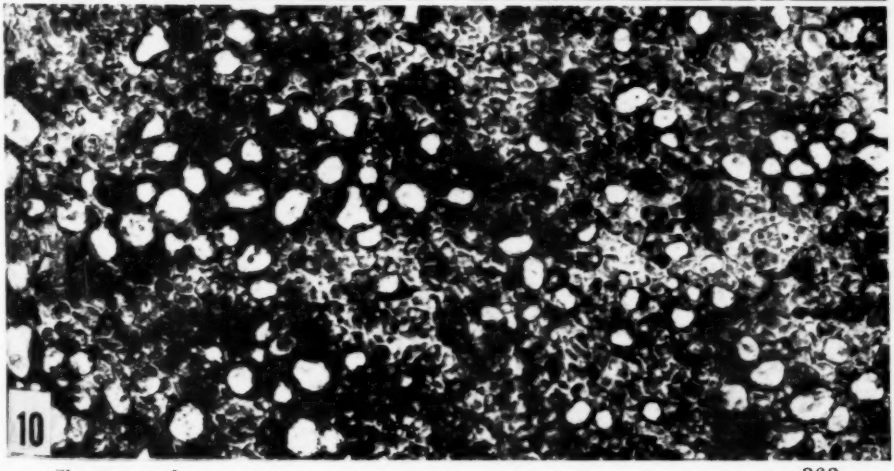
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Damage Induced in Sarcoma 37 with Chemical Agents. II. Trivalent and Pentavalent Arsenicals [1, 2, 3]

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During the past 150 years arsenicals have been tried from time to time in the treatment of malignant disease in patients, and more recently in experimental animals. Dyer's recent "Index of Tumor Chemotherapy" (*3*) lists 178 published items on arsenicals. The earliest ones in this listing were published in 1806 and 1815; many appeared as recently as the last decade. As far as parenteral administration of arsenicals in the treatment of cancer is concerned, the results were considered disappointing.

About 30 years ago Wätjen (*4*, *5*) described the karyoklastic effects of inorganic arsenicals on cells of lymphatic tissues. Dustin and coworkers (*6*, *7*), using chiefly sodium cacodylate, studied the mitotic abnormalities produced by arsenicals in certain normal tissues and in Sarcoma 180. The question arose as to whether screening of a substantial number of arsenicals against Sarcoma 37 might yield information on types of arsenicals worthy of further study as regards their effects on tumors.[5] Sodium cacodylate, the first arsenical we examined, affected Sarcoma 37 only at a dose level which was fatal to some of the mice. Nevertheless, other arsenicals were screened. This paper records the data obtained.

MATERIALS AND METHODS

Almost one hundred organic arsenic compounds, synthesized in our laboratory or obtained from various sources as listed below, were ex-

[1] Received for publication May 12, 1952.

[2] A preliminary report (*1*) of these results was given at the 1948 Annual Meeting of the American Association for Cancer Research.

[3] The first paper (*2*) in this series was entitled "Damage induced in Sarcoma 37 with podophyllin, podophyllotoxin, *alpha*-peltatin, *beta*-peltatin and quercetin."

[4] National Institutes of Health, Public Health Service, Federal Security Agency.

[5] See pages 237 and 239 of a previous publication (*3*) on the scope of this program, including arsenicals.

amined. In the tables, the origin of each preparation is indicated by one of the following code letters:

Code letter	*Source of compound*
A.....	Dr. J. M. Johnson, National Cancer Institute
B.....	Dr. H. Bauer, National Institute of Arthritis & Metabolic Diseases
C.....	Dr. H. Steinman, National Institute of Microbiology
D.....	Dr. R. Hann (deceased), National Institutes of Health
E.....	Chemical-Biological Coordination Center (CBCC), National Research Council, Washington, D. C.
F.....	Abbott Laboratories, Chicago, Illinois
G.....	Eastman Kodak Co., Rochester, N. Y.
H.....	Edcan Co., South Norwalk, Conn.
I.....	Eli Lilly Co., Indianapolis, Ind.
J.....	Hach Chemical and Oxygen Co., Ames, Iowa
K.....	Meister, Lucius and Bruning, Hoechst am Main, Germany
L.....	Merck and Co., Rahway, N. J
M.....	Parke, Davis and Co., Detroit, Mich.
N.....	Squibb Institute for Medical Research, New Brunswick, N. J
O.....	Winthrop Chemical Co., Rensselaer, N. Y

The compounds prepared in our own laboratory were checked for purity by chemical analysis. Those obtained from other sources were, as a general rule, not purified further. With some compounds, the same specimen was examined for maximum tolerated and minimum effective dose (MTD and MED) at various intervals in the course of a year or more. The biological data yielded by several specimens suggested that they had altered in their properties on standing; these instances are shown in the tables. Chemical analysis was of little value in detecting whether deterioration had occurred; as Banks (*9*) has pointed out, the criteria for establishing the purity of even fresh preparations of arsenicals leave much to be desired.[6]

Many of the arsenicals were soluble in water, or formed water-soluble hydrochlorides or sodium salts in dilute aqueous acid or alkali. Those which were insoluble in aqueous medium were dispersed as fine suspensions in olive oil. All solutions or suspensions were freshly prepared just prior to injection.

In this paper the arsenicals are designated as either aliphatic or aromatic, depending upon whether the arsenic is linked to carbon in an aliphatic chain or in an aromatic ring.

[6] For a further consideration of the influence of the purity of arsenical compounds upon biological activity and toxicity, and of the difficulty in obtaining pure specimens, particularly of those arsenicals which exist in several states of molecular aggregation, the reader is referred to the recent paper of George O. Doak and Harry Eagle entitled "Correlations Between the Chemical Structure and Biological Activity of Arsenobenzenes" (in the First Symposium on Chemical-Biological Correlation, Chemical-Biological Coordination Center, National Research Council, Washington, D. C., pp. 7-43, 1951).

To obtain a basis for selection of suitable dose levels for tumor-bearing mice, preliminary toxicity determinations were first performed in a small number of non-tumor-bearing mice, and the mortality observed over a five-day period. The tumor-bearing mice were CAF_1 hybrids with six-day-old implants of Sarcoma 37 in the right thigh muscle. A single dose of each chemical was injected subcutaneously in the left flank. The concentration selected for each agent was such that the desired dose was usually contained in 0.01 to 0.02 cc. for each gram of body weight. The tumors were examined grossly for evidence of induced damage and were later evaluated histologically to supplement the gross observations. The details of the techniques have been described previously (*2*).

In the early years of the screening program in this laboratory, tumor-bearing mice were sacrificed, in groups of about 5 each, at intervals of 8, 24, and 48 hours after a single injection; a similar number of untreated mice bearing tumors implanted from the same mash were killed at these same times as controls. After several hundred compounds were examined in this way, it was seen that the information sought with this type of screening was, in most instances, revealed by the group killed at 24 hours; thereupon, the compounds examined subsequently were evaluated by the effects seen in the tumors 24 hours after injection. Observations at this time interval were obtained with all the compounds reported in this paper; with some of the compounds these data are supplemented with 8- and 48-hour observations.

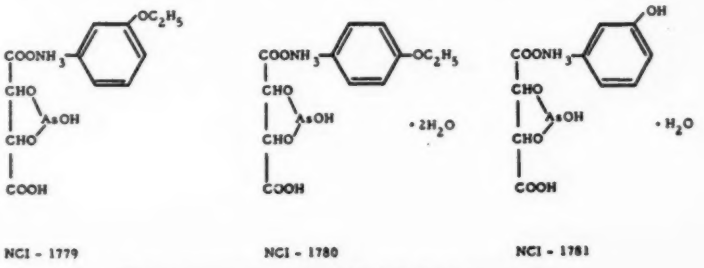
RESULTS

Aliphatic Arsenicals—Pentavalent and Trivalent

With sodium cacodylate, negative results were obtained at 1000 μg. per gm.; however, at 1500 μg. per gm., damage in Sarcoma 37 was induced. Thereupon 5 other aliphatic pentavalent arsenicals were investigated.

Table 1 summarizes the results. Of the five arsonic acids, the methyl- and benzyl- derivatives were negative. The other three aliphatic arsenicals produced damage in Sarcoma 37, but only at doses which produced death in some of the animals.

Three trivalent aliphatic arsenicals were also examined. These were all arsenyl tartrate derivatives. Text-figure 1 and table 2 give the formulas and summarize the findings. The three arsenyl tartrates produced



TEXT-FIGURE 1.—Aliphatic trivalent arsenicals.

TABLE 1.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of aliphatic pentavalent arsenicals*

NCI No.	Compound	Source	Dose (μg./gm.)	Mortality*	Induced tumor damage† at:		
					8 hrs.	24 hrs.	48 hrs.
5v	Sodium cacodylate‡ $(CH_3)_2AsO(ONa)$	L	1500	2/15	5/5	3/5	1/3
			1000	0/15	0/5	0/5	0/5
276	*n*-Propylarsonic acid§ $n\text{-}C_3H_7AsO(OH)_2$	G	‖250	14/23	¶3/3	6/6	.....
			200	0/15	0/5	0/5	0/5
310	Benzylarsonic acid§ $C_6H_5CH_2AsO(OH)_2$	G	150	5/15	0/5	0/4	0/1
313	Ethylarsonic acid§ $C_2H_5AsO(OH)_2$	G	‖100	13/16	.....	3/3	.....
			80	2/8	.....	6/6	.....
			60	0/8	.....	0/8	.....
			50	0/15	0/5	0/5	0/5
367	Sodium methylarsonate‡ $CH_3AsO(ONa)_2$	H	‖1000	0/30	0/10	0/10	0/10
779	Allylarsonic acid§ $C_3H_5AsO(OH)_2$	G	100	3/8	.....	5/5	.....
			80	3/8	.....	4/5	.....
			75	0/7	.....	0/7	.....
			60	0/15	0/5	0/5	0/5

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡Aqueous solution.

§Aqueous alkaline solution.

‖Two separate experiments.

¶Effect seen only microscopically (early cell damage).

TABLE 2.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of trivalent arsenyl tartrates*

NCI No.	Compound (in aqueous solution)	Source	Dose (μg./gm.)	Mortality*	Induced tumor damage† at:		
					8 hrs.	24 hrs.	48 hrs.
1779	*m*-Phenetidine arsenyl tartrate	D	‡40	12/16	.....	4/4	.....
			‡30	3/16	.....	9/13	.....
			25	1/8	.....	2/7	.....
1780	*p*-Phenetidine arsenyl tartrate	D	‡40	12/16	.....	4/4	.....
			30	0/8	.....	3/8	.....
			25	0/8	.....	4/8	.....
1781	*m*-Aminophenol arsenyl tartrate	D	§30	0/24	.....	22/24	.....
			20	0/8	.....	3/8	.....

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡Two separate experiments.

§Three separate experiments.

damage in Sarcoma 37, at doses in the vicinity of the maximum tolerated, as was the case with the pentavalent aliphatic compounds.

In an analogous series of antimonyl tartrates, similar damage was induced in Sarcoma 37; these results will be described in a separate report.

Aromatic Arsenicals—Pentavalent

Forty-six pentavalent aromatic arsenicals, many having trypanocidal activity or other therapeutic properties, were examined for ability to damage Sarcoma 37. Chemically, they fell into two general structural types: 1) *phenyl arsonic acids*, $C_6H_5AsO(OH)_2$; and 2) *phenyl arsinic acids*, $C_6H_5(R)AsO(OH)$. Only two of them produced damage in Sarcoma 37. *Ethyl-4-acetaminophenylarsinic acid* (NCI 245) was active, but only at doses well above the maximum tolerated (6 of 8 mice died at a dose of 300 μg. per gm., and 5 of 6 died at 350 μg. per gm.). The other positive compound, *ethyl-4-iodophenylarsinic acid* (NCI 98v), induced damage in the tumors in 12 of 15 mice at a dose level of 20 μg. per gm. in two separate experiments, in which only one animal died; a slightly lower dose (15 μg. per gm.) was negative. The 44 compounds which did not produce damage in Sarcoma 37 are listed in table 3 with the code number, source, and the highest dose given.

Table 3.—*Pentavalent aromatic arsenicals negative against Sarcoma 37 following a single subcutaneous injection*

NCI No.	Compound (in aqueous vehicle)	Source	Dose* (μg./gm.)	Mortality†
	Arsonic acid			
119	3-Acetamino-4-hydroxyphenyl-	A	1000	0/15
12v	3-Acetamino-4-methoxyphenyl-	A	1000	0/8
189	Arsenacetin	K	1000	0/8
19x	Tryparsamide	L	1000	0/8
200	4-Acetamino-2-hydroxybenzene-	M	1000	0/7
201	Melarsen	M	800	0/7
208	Atoxyl	O	500	0/6
22x	3-Amino-4-hydroxybenzene- ,HCl	M	1000	0/7
220	4-Amino-2-hydroxybenzene-	M	700	0/8
221	4-Carbethoxyamino-2-hydroxybenzene-	M	200	0/15
222	Sodium salt, 3-amino-4-(β-hydroxyethoxy)-phenyl-	M	1000	0/7
223	Phenarsone sulfoxylate (Aldarsone)	F	1000	0/8
225	Carbarsone	I	1000	0/8
242	4-Carboxyphenyl-	A	200	3/15
248	2,5-Dimethoxyphenyl-	A	125	0/8
249	2-Nitrophenyl-	A	1000	0/8
25v	3-Nitrophenyl-	A	40	0/8
25x	3-Methoxyphenyl-	A	50	0/8
314	*p*-Hydroxyphenyl-	G	1000	4/15
338	*p*-Dimethylaminoazophenyl-	G	25	0/15
454	Phenyl-	G	30	0/15
849	4-Nitrophenyl-	B	20	0/8
905	4-Arsono-5-nitrotoluene	A	1000	6/8
906	4-Arsono-5-aminotoluene	A	50	0/8
908	2-Arsonoiodobenzene	A	15	0/8
976	2-Arsono-5-iodoanisole	A	200	0/8

See footnotes at end of table.

TABLE 3.—*Pentavalent aromatic arsenicals negative against Sarcoma 37 following a single subcutaneous injection*—Continued

NCI No.	Compound (in aqueous vehicle)	Source	Dose* (μg./gm.)	Mortality†
	Arsonic acid—Continued			
978	3-Arsonopyridine	A	300	1/8
1128	3-Iodo-4-aminophenyl-	A	50	0/8
1129	3-Iodo-4-hydroxyphenyl-	A	50	0/15
1130	3-Iodo-4-methoxyphenyl-	A	40	5/15
1132	3-Nitro-4-hydroxyphenyl-	A	400	6/17
1246	Phenazine-2,7-bis-	A	100	0/15
2446	Thiolcarbamic acid, N-mercaptoacetylarsanilic acid ester‡	E	200	.....
	Arsinic acid			
243	Methyl-4-carboxyphenyl-	A	50	0/8
244	Methyl-4-acetaminophenyl-	A	250	0/8
246	Methyl-3-acetamino-4-hydroxyphenyl-	A	300	0/8
247	Ethyl-3-acetamino-4-hydroxyphenyl-	A	500	0/8
814	*n*-Heptyl-3-acetamino-4-hydroxyphenyl-	A	100	0/3
815	Benzyl-3-acetamino-4-hydroxyphenyl- §	A	5	0/15
816	Isoamyl-3-acetamino-4-hydroxyphenyl-	A	50	0/15
82v	Methyl-3-carboxyphenyl-	A	400	0/15
823	Methyl-3-nitrophenyl-	A	30	0/15
846	4-Nitro-4′-acetylaminodiphenyl-	B	150	0/8
847	4-Nitro-4′-aminodiphenyl-	B	80	0/8

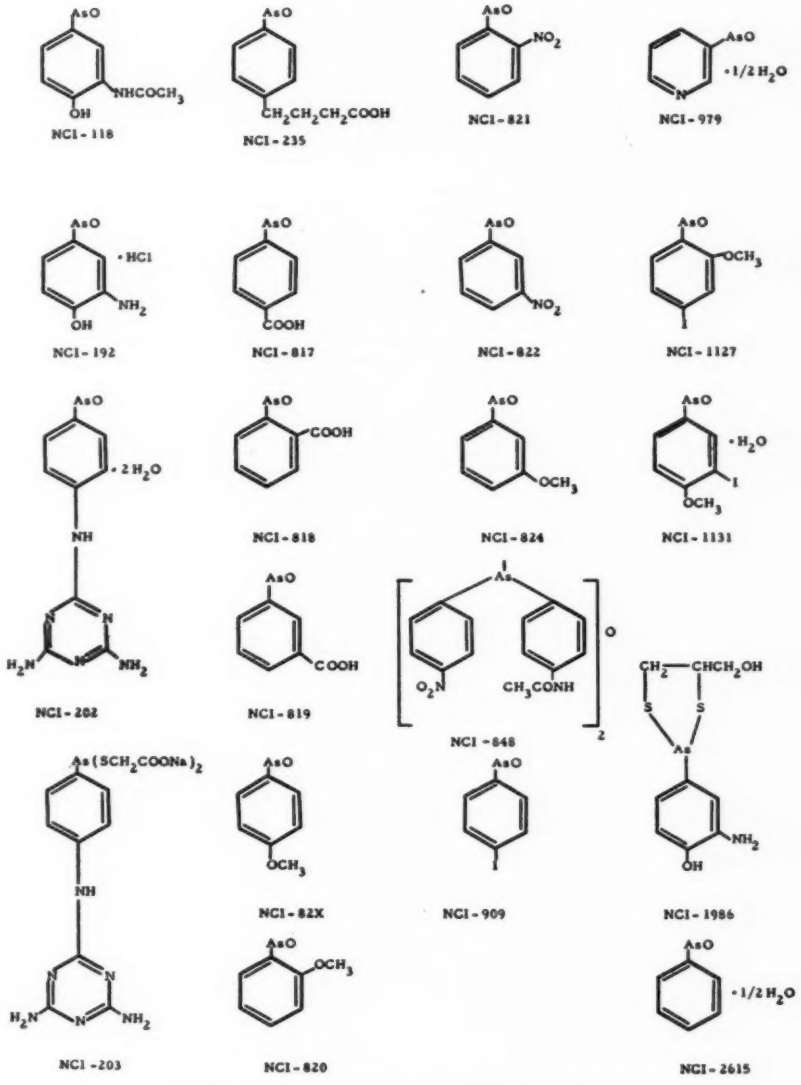
* Highest dose given.
† No. of mice dead. / No. of mice treated
‡ Unpublished work of Belkin and Fitzgerald.
§ Olive oil suspension.

AROMATIC ARSENICALS—TRIVALENT

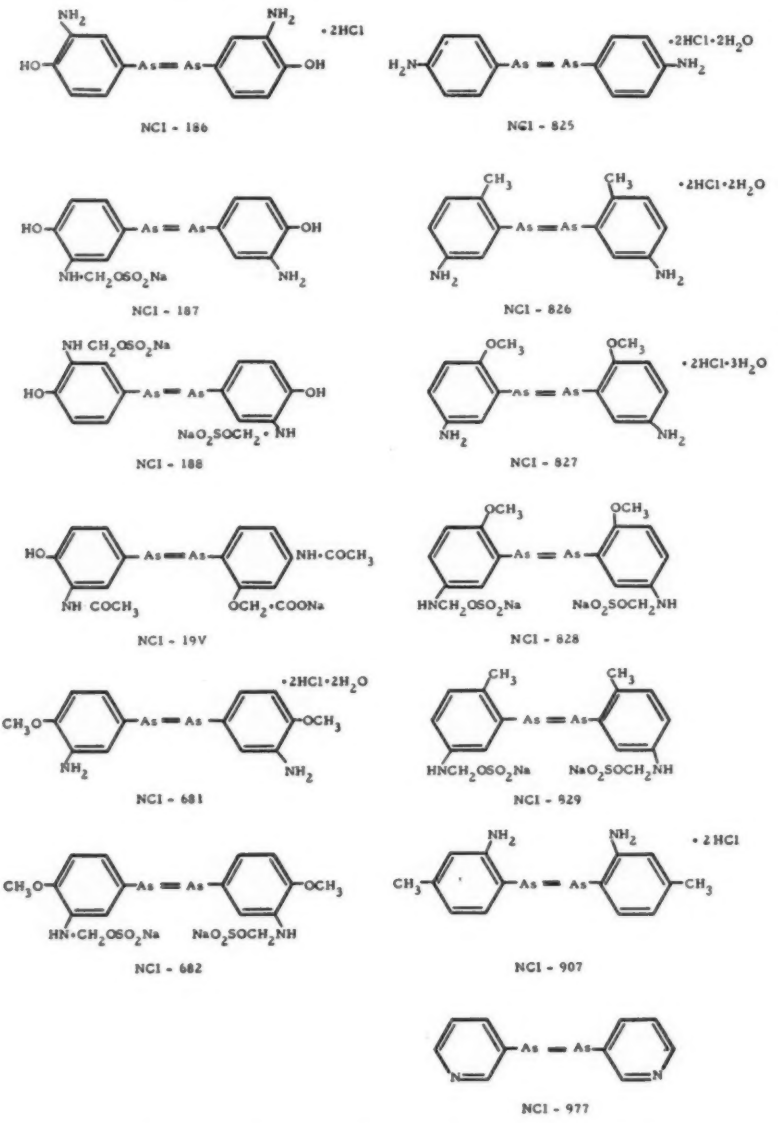
The 36 arsenic compounds in this class consisted of three general types: 1) *arsenoxides*, R-As=O; 2) *arseno compounds*, R-As=As-R; and 3) *arsines*, R_3As.

In contrast with the pentavalent aromatic compounds, the trivalent ones yielded a large number of compounds capable of producing hemorrhage and necrosis in Sarcoma 37 following a single subcutaneous injection. Text-figures 2 to 4, and tables 4 to 6, give the formulas of the compounds and summarize the effects obtained. Of these trivalent arsenicals, 27 produced damage in the tumors at one or more dose levels. As with the aliphatic arsenicals, the doses producing consistent damage in the tumors were all near the maximum tolerated.

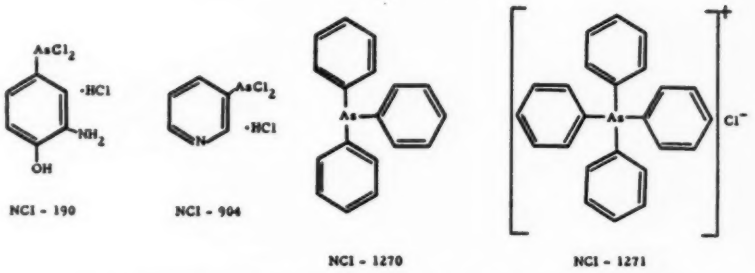
Table 7 lists the positive arsenic compounds with approximate values for the maximum tolerated dose (MTD) and the minimum effective dose (MED) of each, determined as described earlier (*2*). In no instance was a high value obtained for the ratio MTD/MED.



TEXT-FIGURE 2.—Aromatic arsenoxides (*trivalent*).



TEXT-FIGURE 3.—Aromatic arseno compounds (*trivalent*).



TEXT-FIGURE 4.—Arsines (*trivalent, including one arsonium salt*).

TABLE 4.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of trivalent arsenoxides*

NCI No.	Compound	Source	Dose (μg./gm.)	Mortality*	Induced tumor damage† at:		
					8 hrs.	24 hrs.	48 hrs.
118	3-Acetamino-4-hydroxyphenylarsenoxide‡	A	§35	4/29	7/9	3/10	0/6
			30	0/8	.....	3/8	.....
			20	0/8	.....	0/8	.....
192	3-Amino-4-hydroxyphenylarsenoxide HCl (Mapharsen)‖	M	§30	0/23	4/5	11/13	5/5
			25	0/15	0/5	3/5	0/5
			20	0/8	.....	0/8	.....
202	Melarsen oxide, dihydrate¶	M	70	8/8	.....	.....	.....
			50	0/8	.....	7/8	.....
			§30	1/16	.....	9/15	.....
203	Melarsen oxide dithioglycolate‖	M	§100	9/16	.....	6/7	.....
			80	2/8	.....	2/6	.....
			60	0/8	.....	0/8	.....
235	γ-(*p*-Arsenosophenyl)-*n*-butyric acid‡	C	40	6/8	.....	1/2	.....
			§30	0/16	.....	2/16	.....
817	4-Arsenosobenzoic acid‡	A	30	0/8	.....	0/8	.....
			25	0/8	.....	0/8	.....
818	2-Arsenosobenzoic acid‡	A	10	7/8	.....	1/1	.....
			8	4/8	.....	3/4	.....
			§5	0/16	.....	10/16	.....
			3	0/8	.....	1/8	.....
819	3-Arsenosobenzoic acid‡	A	30	3/8	.....	0/5	.....
			25	0/8	.....	0/8	.....
82x	4-Methoxyphenylarsenoxide‖	A	10	6/8	.....	2/2	.....
			8	3/7	.....	3/4	.....

See footnotes at end of table.

TABLE 4.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of trivalent arsenoxides*—Continued

NCI No.	Compound	Source	Dose (μg./gm.)	Mortality*	Induced tumor damage† at:		
					8 hrs.	24 hrs.	48 hrs.
820	2-Methoxyphenylarsenoxide**	A	50	0/8	0/5	0/5	0/5
821	2-Nitrophenylarsenoxide‡	A	120	0/8	.....	0/8	.....
			100	0/8	.....	0/8	.....
822	3-Nitrophenylarsenoxide‡	A	3	0/8	.....	8/8	.....
			§2	0/16	.....	9/16	.....
			1.5	0/8	.....	2/8	.....
824	3-Methoxyphenylarsenoxide**	A	10	0/8	.....	4/8	.....
			7	1/15	0/5	5/5	4/4
			6	0/8	.....	0/8	.....
848	bis(4-Nitro-4′-acetaminodiphenylarsenic)oxide‡	B	150	8/8	.....	.....	.....
			100	0/8	.....	2/8	.....
			60	0/8	.....	0/8	.....
909	4-Iodophenylarsenoxide‡	A	25	2/8	.....	3/6	.....
			20	0/8	.....	0/8	.....
			§15	0/16	.....	1/16	.....
979	3-Arsenosopyridine, hemihydrate‖	A	5	2/8	.....	6/6	.....
			§2	2/16	.....	7/14	.....
1127	2-Methoxy-4-iodophenylarsenoxide**	A	30	2/8	.....	6/6	.....
			20	0/8	.....	6/8	.....
			§15	2/15	.....	6/13	.....
1131	3-Iodo-4-methoxyphenylarsenoxide‡	A	10	2/8	.....	6/6	.....
			6	0/8	.....	8/8	.....
			§3	0/16	.....	2/16	.....
1986	BAL-OXO ¶	A	30	6/8	.....	1/2	.....
			15	5/16	.....	2/11	.....
2615	Phenylarsenoxide‡	A	1.5	3/8	.....	5/5	.....
			1	1/8	.....	0/7	.....

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡Aqueous alkaline solution.
§Two separate experiments.
‖Aqueous solution.
¶Aqueous acid solution.
**Olive oil suspension.

TABLE 5.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of trivalent arsenocompounds*

NCI No.	Compound*	Source	Dose (μg./gm.)	Mortality†	Induced tumor damage‡ at:		
					8 hrs.	24 hrs.	48 hrs.
907	2,2′-Diamino-4,4′-dimethylarsenobenzene 2HCl	A	40	4/16	.....	11/12	.....
			25	0/8	.....	4/8	.....
186	Arsphenamine.....	O	100	0/8	.....	0/8	.....
			75	0/8	0/3	0/3	0/2
187	Neoarsphenamine.....	N	225	3/8	.....	5/5	.....
			175	2/8	.....	2/6	.....
			150	1/8	0/2	0/3	0/2
188	Sulfarsphenamine.....	N	200	3/8	.....	5/5	.....
			150	0/15	3/5	3/5	2/5
19v	Acetylglycarsenobenzene (Solu-Salvarsan)	O	1000	0/8	0/3	0/3	0/2
681	3,3′-Diamino-4,4′-dimethoxyarsenobenzene 2HCl·$2H_2O$	A	30	1/8	.....	5/7	.....
			§25	0/16	.....	6/16	.....
682	3,3′-Diamino-4,4′-dimethoxyarsenobenzene-N,N′-dimethylenesulfonate disodium salt	A	175	8/8	.....	.....	.....
			125	6/8	.....	0/2	.....
			100	0/8	0/3	0/3	0/2
825	4,4′-Diaminoarsenobenzene dihydrochloride	A	40	2/8	.....	6/6	.....
			30	1/8	.....	6/7	.....
826	5,5′-Diamino-2,2′-dimethylarsenobenzene 2HCl·$2H_2O$	A	25	3/17	5/5	3/4	1/5
			10	0/8	.....	6/8	.....
			5	0/8	.....	0/8	.....
827	5,5′-Diamino-2,2′-dimethoxyarsenobenzene 2HCl	A	25	0/8	.....	5/8	.....
			§15	0/16	.....	3/16	.....
828	5,5′-Diamino-2,2′-dimethoxyarsenobenzene-N,N′-dimethylenesulfonate disodium salt	A	15	2/15	3/3	0/4	3/3
			10	0/15	0/5	0/5	0/5
829	5,5′-Diamino-2,2′-dimethylarsenobenzene-N,N′-dimethylenesulfonate disodium salt	A	40	1/8	.....	7/7	.....
			30	0/8	.....	6/8	.....
			20	0/15	3/5	0/5	0/5
977	3,3′-Arsenopyridine.....	A	7.5	4/8	.....	4/4	.....
			§5	0/16	.....	2/16	.....

*Aqueous vehicle used for all compounds except NCI 825.

†Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

‡Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

§Two separate experiments.

TABLE 6.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of other arsenicals (arsines; arsonium salt)*

NCI No.	Compound	Source	Dose (μg./gm.)	Mortality*	Induced tumor damage† at:		
					8 hrs.	24 hrs.	48 hrs.
190	3-Amino-4-hydroxyphenyldichlorarsine HCl (Chlorarsen)‡	N	§30	6/71	17/20	23/29	13/16
			25	0/15	4/5	0/5	0/5
			20	0/8	.....	0/8	.....
904	3-Pyridyldichlorarsine HCl ‡	A	‖5	3/16	.....	11/13	.....
			3	0/8	.....	6/8	.....
1270	Triphenylarsine¶.....	J	700	0/8	.....	0/8	.....
1271	Tetraphenylarsonium chloride¶	J	80	5/8	.....	0/3	.....
			60	4/8	.....	0/4	.....

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice treated}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated controls showed no induced damage.

‡In aqueous solution.
§Six separate experiments.
‖Two separate experiments.
¶Olive oil suspension.

TABLE 7.—*Approximate values of the maximum tolerated dose (MTD) and minimum effective dose (MED) of arsenicals which produced damage in Sarcoma 37*

NCI No.	Compound	MTD (μg./gm.)	MED (μg./gm.)
	Pentavalent aliphatic (6 tested)		
5v	Sodium cacodylate.....	1500	1500
276	*n*-Propylarsonic acid.....	200	250
313	Ethylarsonic acid.....	80	80
779	Allylarsonic acid.....	80	80
	Trivalent aliphatic (3 tested)		
1779	*m*-Phenetidine arsenyl tartrate.....	25	20
1780	*p*-Phenetidine arsenyl tartrate.....	30	20
1781	*m*-Aminophenol arsenyl tartrate.....	30–40	20
	Pentavalent aromatic (46 tested)		
245	Ethyl-4-acetaminophenylarsinic acid.....	250	300
98v	Ethyl-4-iodophenylarsinic acid.....	20	20
	Trivalent aromatic		
	Arsenoxides (20 tested)		
118	3-Acetamino-4-hydroxyphenylarsenoxide.....	35	30
192	Mapharsen.....	30–40	25
202	Melarsen oxide.....	35	25
203	Melarsen oxide dithioglycolate.....	80	80
235	γ-(*p*-Arsenosophenyl)-*n*-butyric acid.....	30–40	30
818	2-Arsenosobenzoic acid.....	6	4
82x	4-Methoxyphenylarsenoxide.....	8	8
822	3-Nitrophenylarsenoxide.....	3.0	*1.5
824	3-Methoxyphenylarsenoxide.....	7	7
848	bis(4-Nitro-4′-acetylaminodiphenylarsenic)oxide...	125	100
979	3-Arsenosopyridine.....	2	2
1127	2-Methoxy-4-iodophenylarsenoxide.....	15	15
1131	3-Iodo-4-methoxyphenylarsenoxide.....	6	3
1986	BAL–OXO.....	15	15
2615	Phenylarsenoxide.....	1	1.5

See footnotes at end of table.

TABLE 7.—*Approximate values of the maximum tolerated dose (MTD) and minimum effective dose (MED) of arsenicals which produced damage in Sarcoma 37*—Continued

NCI No.	Compound	MTD (μg./gm.)	MED (μg./gm.)
	Arseno compounds (13 tested)		
187	Neoarsphenamine	175	175
188	Sulfarsphenamine	150	150
681	3,3′-Diamino-4,4′-dimethoxyarsenobenzene	25	15
825	4,4′-Diaminoarsenobenzene	30	†20
826	5,5′-Diamino-2,2′-dimethylarsenobenzene 2HCl·$2H_2O$	20	20
827	5,5′-Diamino-2,2′-dimethoxyarsenobenzene 2HCl	40	20
828	5,5′-Diamino-2,2′-dimethoxyarsenobenzene-N, N′-dimethylene disodium sulfonate	15	†15
829	5,5′-Diamino-2,2′-dimethylarsenobenzene-N, N′-dimethylene disodium sulfonate	30–40	20
907	2,2′-Diamino-4,4′-dimethylarsenobenzene	30	25
977	3,3′-Arsenopyridine	7.5	5
	Arsines; Arsonium salt (4 tested)		
190	Chlorarsen	30	30
904	3-Pyridyldichloroarsine HCl	4	3

*Value given by Beck (*10*) was obtained when drug was administered in an olive oil vehicle. Our results are for an aqueous vehicle.

†Different values were obtained at different times with the same and with different specimens (*cf.* Materials and Methods).

DISCUSSION

This paper presents data on the potency of 92 arsenic compounds in damaging Sarcoma 37 following a single subcutaneous injection. These compounds included aliphatic and aromatic derivatives containing trivalent or pentavalent arsenic. Of the pentavalent aliphatic arsenicals, 4 of the 6 examined yielded positive results. Only 2 pentavalent aromatic arsenicals of the 46 investigated had potency, whereas 27 of the 36 trivalent aromatic arsenicals were active.

A dose in the vicinity of the maximum tolerated was required for production of tumor damage in the case of all of the positive compounds reported here. It was emphasized in the first paper of this series (*2*) that numerous factors cause both MTD and MED in tumor-bearing mice to vary; hence such values represent only approximations. Conceivably, some compounds found to be negative at the highest dose given might yield positive results at a slightly higher one. There were, in fact, several arsenicals which gave questionable or no evidence of damage in the tumors at a dose level near the MTD, but which yielded positive results when repeated at the same or a slightly higher dose. In some instances, the highest dose employed caused no deaths in the animals, with negative tumor results; if a somewhat higher dose, closer to the MTD, were tried it might produce damage in the tumors. It was not considered worthwhile, however, to perform additional experiments with these "negative" compounds inasmuch as low values would be obtained for the ratio MTD/MED even if a bigger dose should yield positive results. Insofar as these data go, the trivalent arsenicals were preponderantly positive and the pentavalent ones preponderantly negative.

The narrow range between MTD and MED exhibited by these compounds does not necessarily preclude consideration of them for further work in experimental animals or patients. A substantial number of them have, or have had, extensive clinical use in other diseases, and considerable information is available on dose tolerance and pharmacologic effects. Counteraction of the toxicity of arsenicals by BAL has been established. Beck (*10*) has found that protection against death was obtained with BAL even when it was given five hours after injection of certain of these arsenicals. Our own investigations (*2*) have shown that tumor-damaging compounds, including arsenicals, are capable of inducing damage in as little as two hours. Variations in the timing of administration of arsenicals and of BAL may reveal conditions under which tumor-damaging potency might be retained with less hazard to the host.

SUMMARY

Ninety-two arsenicals were examined, in over 3,000 tumor-bearing mice, for potency in damaging Sarcoma 37 following a single subcutaneous injection. The compounds were aliphatic and aromatic derivatives containing pentavalent or trivalent arsenic. The following results were obtained:

Derivatives	*No. examined*	*No. positive*
aliphatic-pentavalent	6	4
aliphatic-trivalent	3	3
aromatic-pentavalent	46	2
aromatic-trivalent	36	27

A dose near the maximum tolerated was required for production of tumor damage with all of the active compounds.

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Damage Induced in Sarcoma 37 with Chemical Agents. III. Colchicine Derivatives Related to Trimethylcolchicinic Acid and to Colchinol [1,2]

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Although the first report on the effect of colchicine upon cell division has generally been attributed to Dixon (*3*) at the beginning of this century, Eigsti, Dustin Jr., and Gay-Winn (*4*) have recently pointed out that, as far back as 1889, Pernice (*5*) had observed mitotic abnormalities in gastric and intestinal mucosa of dogs treated with extracts of colchicum corms. However, it was not until 1934 that its powerful action as a mitotic poison was extensively studied, by Dustin (*6*) and Lits (*7*); its application to the treatment of animal tumors was first reported in 1935 by Amoroso (*8*). A brief review of its origin and of its use in medicine in ancient and medieval times was given by Sharp in 1909 (*9*). Chodkowski (*10*) reviewed the work on its effects upon mitosis in 1937. More recently Ludford (*11*) has summarized the earlier literature, especially with respect to the use of colchicine against animal and human tumors. Within the past few years investigators have explored its action on cell division by counting mitoses in such varied material as gastric mucosa (*12*), Sarcoma 180 (*13*), corneal epithelium (*14*), and spermatogonia (*15*); these studies were concerned particularly with correlating mitotic activity with the chemical structure of colchicine and a few of its derivatives. Despite the high toxicity of the drug and the disappointing results of clinical trials, interest in it has continued. The hypothesis that suitable modification of the structure might retain the tumor-inhibiting activity while reducing systemic toxicity has remained an attractive one.

The chemical structure of colchicine is still not completely established. Windaus (*16*) originally proposed a reduced phenanthrene nucleus for the basic structure (text-fig. 1). However, later investigators (*17–22*) have presented convincing evidence that one, or both, of the B and C rings of colchicine are seven-membered; the structure favored currently

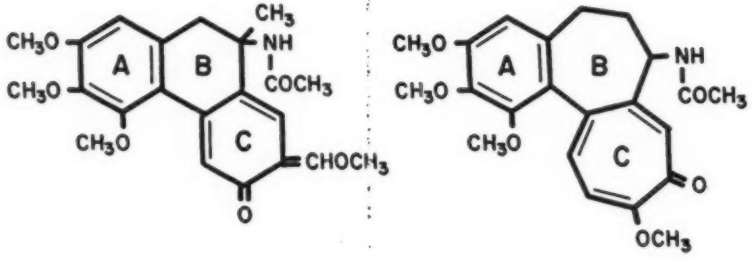
[1] Received for publication May 12, 1952.

[2] Preliminary reports (*1, 2*) on these agents were presented, in part, at the Annual Meeting of the American Association for Cancer Research in 1949 and the Fourth International Cancer Congress.

[3] National Institutes of Health, Public Health Service, Federal Security Agency.

is shown in text-figure 1. In particular, the total synthesis of *dl*-colchinol methyl ether by Rapoport and coworkers (*21*) has established the certainty of a seven-membered B ring. Recent work on the structure of colchicine has made available derivatives of colchicine with varying degrees of modification of the basic three-ring structure, so that a more extensive investigation of the relationship of chemical structure to biological activity could be made.

COLCHICINE



Windaus Formula **Dewar-Cook-Tarbell Formula**

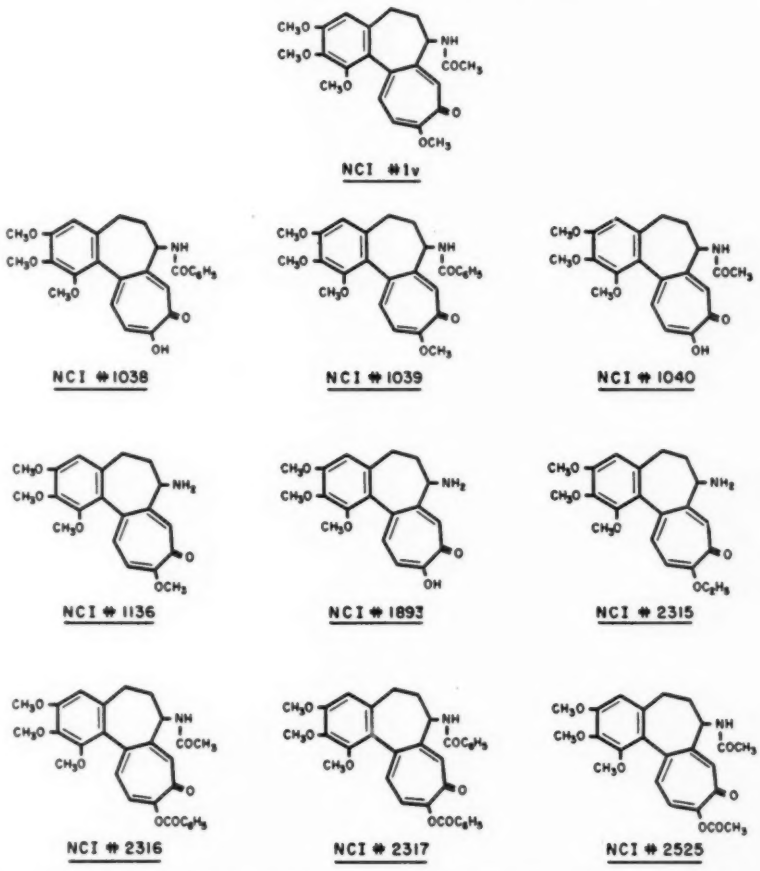
TEXT-FIGURE 1.—Chemical structure of colchicine. On the left is the old formula of Windaus; on the right, the formula favored currently.

As part of the systematic program of developing chemical agents for investigations in the chemotherapy of cancer (*23*), work on this group of compounds was undertaken in 1944 in this Laboratory. Some of the material presented here has been the subject of preliminary reports cited above (*1*, *2*). In the experiments reported in this paper, 16 compounds were studied in about 1500 mice bearing Sarcoma 37.

MATERIALS AND METHODS

Colchicine, U. S. P., was obtained from commercial sources. From the light brown solid, after purification by chromatographing (*24*) on activated alumina, a white crystalline product of high purity was obtained. Unless otherwise indicated in the text, the derivatives of colchicine studied were prepared by Dr. Glenn E. Ullyot and associates. Text-figures 2 and 3, and table 1, give formulas and melting points of the compounds.

The derivatives of colchicine fell into two general classes. The first group (text-fig. 2) retained the basic ring structure of colchicine (NCI No. 1v) with both B and C rings seven-membered; these compounds are considered as derivatives of *trimethylcolchicinic acid* (NCI No. 1893). Thus colchiceine and colchicine are termed N-acetyltrimethylcolchicinic acid (NCI No. 1040) and its methyl ether (NCI No. 1v), respectively. Other derivatives represented changes in the substituents on the amino

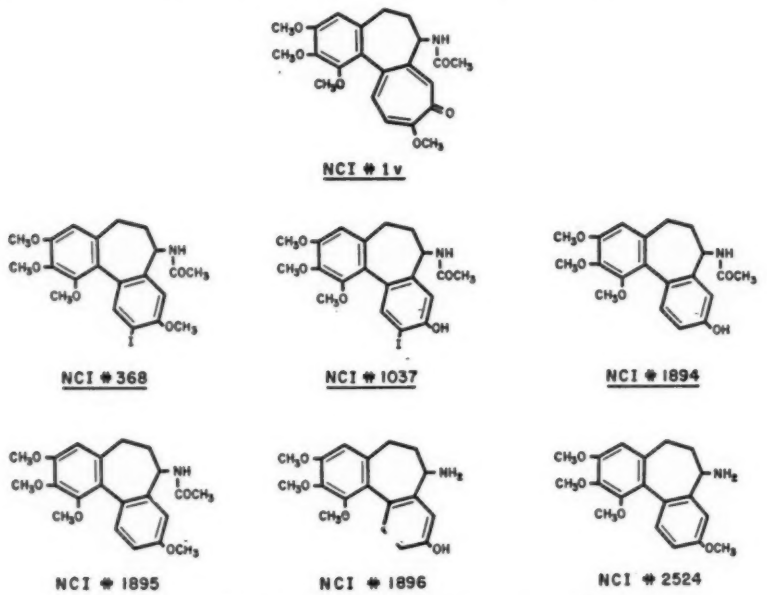


TEXT-FIGURE 2.—Colchicine derivatives from the trimethylcolchicinic acid series.

group of the B ring and/or on the hydroxyl group of the C ring. The hydroxyl group in trimethylcolchicinic acid and in some of its derivatives has acidic properties and, accordingly, they are soluble in dilute sodium hydroxide.

The second group (text-fig. 3) consisted of analogs with a six-membered aromatic C ring; the hydroxyl group in these derivatives is phenolic and therefore soluble in dilute sodium hydroxide. These analogs are considered as aromatized derivatives of colchicine. In the literature, *colchinol* (No. 1896) has been considered the parent substance of such compounds for purposes of nomenclature.

Several of the compounds were water-soluble tartrates or hydrochlorides. Those which were not soluble in aqueous media were injected as suspensions in olive oil.



TEXT-FIGURE 3.—Colchicine derivatives from the colchinol series.

TABLE 1.—*Melting points and solubility in water of the colchicine derivatives examined*

Compound	NCI No.	Melting point* (° C.)	Solubility in aqueous vehicle
Colchicine (N-acetyltrimethylcolchicinic acid methyl ether)	1v	155–158	Very soluble.
N-acetyliodocolchinol methyl ether	368	†129–132	Insoluble.
N-acetyliodocolchinol	1037	229–230	Soluble.‡
N-benzoyltrimethylcolchicinic acid	1038	258–259	Soluble.‡
N-benzoyltrimethylcolchicinic acid methyl ether§	1039	146–155	Insoluble.
Colchiceine (N-acetyltrimethylcolchicinic acid)	1040	177–178	Soluble.‡
Trimethylcolchicinic acid methyl ether d-tartrate	1136	215–216	Soluble.
Trimethylcolchicinic acid	1893	153–156	Soluble.‖
N-acetylcolchinol	1894	153–154; 210–211	Soluble.‡
N-acetylcolchinol methyl ether	1895	205–206	Insoluble.
Colchinol	1896	241.5–242	Soluble.‖
Trimethylcolchicinic acid ethyl ether d-tartrate§	2315	202–204.5	Soluble.
O-benzoyl-N-acetyltrimethylcolchicinic acid	2316	197–199	Insoluble.
O,N-dibenzoyltrimethylcolchicinic acid	2317	292–293	Insoluble.
Colchinol methyl ether hydrochloride	2524	253–255	Soluble.
O,N-diacetyltrimethylcolchicinic acid§	2525	135–145	Insoluble.

*Determined by Drs. Ullyot and Horowitz.

†This melting point was determined by us; an earlier specimen, obtained from Dr. Tarbell, University of Rochester, melted at 117.5–123.0°. The value reported by Windaus (*16*) was 122–123°. According to Dr. Ullyot, the melting points of different specimens varied, but chemical analysis as well as derivatives obtained from them were fully consistent with its identity and purity.

‡In dilute NaOH.

§No attempt was made to separate the two possible isomeric forms; a mixture of the normal and *iso* forms is thus not excluded.

‖In dilute NaOH or HCl.

Hybrid mice (CAF_1 and ABC) were used in these experiments. Approximate values for MTD[4] were determined in non-tumor-bearing mice over a period of five days after a single subcutaneous injection. Mice, principally CAF_1, bearing six-day-old intramuscular transplants of Sarcoma 37, were given a single subcutaneous injection of each compound; groups of five to eight animals at a time were sacrificed at approximately 8, 24, and 48-hour intervals after the injection. Untreated tumor-bearing control mice were sacrificed at the same times for comparison. The tumors were examined for evidence of gross damage (hemorrhage, softening, and necrosis). Hematoxylin-eosin stained sections of the tumors were evaluated histologically (*26*, *27*). Further details on these procedures are given in an earlier paper (*25*).

The data reported here are for mice which were sacrificed; those that were found dead were discarded. Unless otherwise indicated in the tables or text, the data are summaries of the gross findings, which were usually corroborated upon subsequent microscopic examination. When gross damage was seen in the tumors, the histologic findings confirmed the observation. In a few instances histologic examination revealed distorted mitotic figures or arrested mitoses, without necrosis, while the tumors appeared negative grossly. Some of these mitotic changes appeared to be reversible since, in mice sacrificed at later time intervals, neither gross nor miscroscopic evidence of necrosis or other damage was seen.

RESULTS

Representative data, illustrating the results, are described in this report.

Trimethylcolchicinic acid derivatives.—Table 2 summarizes data on the effects of a single subcutaneous injection of colchicine and of each of the trimethylcolchicinic acid derivatives investigated. The type of gross damage observed was similar to that described in some detail in an earlier publication (*25*) on the effects of components of podophyllin. Briefly, hemorrhage and partial liquefaction of the tumors usually occurred within 8 hours after injection, and increased in severity up to 24 to 48 hours. By that time degeneration of the affected tumor tissue, which became discolored, caseous, and necrotic, was evident. Unaffected or newly generated tumor tissue was sometimes visible at the periphery. Complete destruction of the tumors was not usually observed following a single injection.

Microscopically, sections of the treated tumors showed pyknosis of the nuclei, retraction of the cytoplasm, and bizarre mitotic figures; after 24 and 48 hours, induced generalized pyknosis or necrosis, and cellular disintegration were pronounced. These effects were similar to those described previously (*25*).

Of the ten derivatives of trimethylcolchicinic acid, only two did not produce necrosis in Sarcoma 37 in doses up to about 1000 micrograms per gram. These were N-benzoyltrimethylcolchicinic acid (NCI No. 1038)

[4] The maximum tolerated dose (MTD) used in this paper is an approximate value and is used in the same sense as described previously (*25*), *viz.*, "The largest dose in a single injection that caused no deaths (or, at most, deaths in a small percent of the mice)."

TABLE 2.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of colchicine or of other trimethylcolchicinic acid derivatives*

Compound	NCI No.	Vehicle	Dose (μg./gm.)	No. of expts.	Mortality*	Induced tumor damage† at:		
						8 hrs.	24 hrs.	48 hrs.
Colchicine (N-acetyltrimethylcolchicinic acid methyl ether)	1v	Water	3	3	2/25	7/7	12/12	4/4
			1.5	1	0/8	.....	3/8	.....
			1	1	0/7	.....	1/7	.....
N-benzoyltrimethylcolchicinic acid	1038	Aqueous NaOH	920	1	0/15	0/5	0/5	0/5
			700	2	0/20	0/5	‡0/10	0/5
N-benzoyltrimethylcolchicinic acid methyl ether	1039	Olive oil	100	2	8/16	.....	8/8	.....
			80	1	0/8	.....	8/8	.....
			40	1	0/8	.....	‡8/8	.....
Colchiceine (N-acetyltrimethylcolchicinic acid)	1040	Aqueous NaOH	100	2	0/22	5/5	13/13	4/4
			50	2	0/16	.....	‡0/16	.....
Trimethylcolchicinic acid methyl ether d-tartrate	1136	Water	125	1	3/15	4/4	4/4	4/4
			4	2	0/18	.....	18/18	.....
			2	2	0/20	.....	8/20	.....
			1	2	0/20	.....	0/20	.....
Trimethylcolchicinic acid	1893	Aqueous HCl	800	3	6/21	.....	7/15	.....
			600	3	3/21	.....	6/18	.....
			400	1	0/15	0/5	0/5	0/5
Trimethylcolchicinic acid ethyl ether d-tartrate	2315	Water	100	2	1/16	.....	15/15	.....
			3	2	0/16	.....	6/16	.....
			2	2	0/13	.....	0/13	.....
O-benzoyl-N-acetyltrimethylcolchicinic acid	2316	Olive oil	1000	2	0/16	.....	3/16	.....
O,N-dibenzoyltrimethylcolchicinic acid	2317	Olive oil	1000	1	0/8	.....	0/8	.....
O,N-diacetyltrimethylcolchicinic acid	2525	Olive oil	400	1	0/8	.....	8/8	.....
			200	2	0/16	.....	6/16	.....

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated control animals showed no induced damage.

‡Distorted mitotic figures and/or arrested mitoses seen microscopically, but no evidence of induced necrosis seen either grossly or microscopically.

and O,N-dibenzoyltrimethylcolchicinic acid (NCI No. 2317). The vehicles used did not appear to be a factor in these two cases, inasmuch as positive effects were obtained with other derivatives given as a solution in aqueous alkali (*e.g.*, NCI No. 1040) or as a suspension in oil (*e.g.*, NCI No. 1039).

Colchinol derivatives.—Table 3 gives illustrative data on the damage induced in Sarcoma 37 by those derivatives of colchicine in which the C ring was degraded to a six-membered aromatic ring. Damage induced with all six of these compounds was similar to that described above. It is apparent that the C ring need not be seven-membered for the molecule to possess tumor-damaging activity. Like the compounds listed in table 2, the toxicity of those in this group was considerably lower than that of colchicine, and potency was not dependent on whether they were given in aqueous medium or in oil suspension. Two compounds, N-acetylcolchinol (NCI No. 1894) and its methyl ether (NCI No. 1895), produced damage to tumors at dose levels as low as 4 and 5 μg. per gm., respectively. Since these two compounds were tolerated in much larger doses than colchicine, they had a considerably wider spread between the maximum tolerated dose and the minimum effective dose than did colchicine.

Maximum tolerated dose and minimum effective dose.—The maximum tolerated dose (MTD) and minimum effective dose (MED) for each of the active compounds were determined in mice bearing six-day-old implants of Sarcoma 37. Some of the groups which received the higher doses were usually observed for periods up to five days for lethality; those injected with lower doses were sacrificed after 24 hours and the tumors examined in the usual fashion for induced damage. In this way, approximate [5] values for the range between the MTD and MED were obtained for tumor-bearing mice. Table 4 gives the results obtained for MTD and MED, as well as the ratio MTD/MED, for each of the active compounds which induced necrosis in the tumors.

Rather wide differences in the values of the ratio MTD/MED were obtained. Seven compounds, including colchicine, had a ratio of two or less. Six of the compounds had a ratio of four or greater, a value appreciably greater than that for colchicine itself; the ratio for three compounds was 25 or greater. These data fortify the hypothesis that modification of the structure of colchicine can reduce lethality without equivalent loss of tumor-damaging potency as determined by this technique.

The toxic symptoms of all these drugs at their MTD were similar to those produced by colchicine; diarrhea was a common feature in all the mice given high doses.

DISCUSSION

These data indicate that considerable modification of both lethality and tumor-damaging activity may be obtained with rather minor modifications in the colchicine molecule. Thus demethylation of colchicine to colchiceine resulted in an increase in MTD from 3 to 150 as well as a

[5] See footnote 5 in reference (*25*).

TABLE 3.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of colchicine and of colchinol derivatives*

Compound	NCI No.	Vehicle	Dose (μg./gm.)	No. of expts.	Mortality*	Induced tumor damage† at:		
						8 hrs.	24 hrs.	48 hrs.
Colchicine	1v	Water	3	3	2/25	7/7	12/12	4/4
			2	1	0/7	.....	7/7	.....
N-acetyliodocolchinol methyl ether	368	Olive oil	300	4	0/26	8/8	15/15	2/3
			200	2	0/19	3/3	7/16	.....
N-acetyliodocolchinol	1037	Aqueous NaOH	250	2	1/30	10/10	10/10	7/9
			50	2	0/16	.....	8/16	.....
			25	2	0/16	.....	0/16	.....
N-acetylcolchinol	1894	Aqueous NaOH	125	‡3	4/31	.....	11/11	.....
			10	3	0/24	.....	23/24	.....
			4	2	0/16	.....	6/16	.....
N-acetylcolchinol methyl ether	1895	Olive oil	25	‡3	11/31	5/5	4/4	0/1
			15	1	0/8	.....	8/8	.....
			5	2	0/16	.....	14/16	.....
Colchinol	1896	Aqueous HCl	350	1	0/15	5/5	5/5	5/5
			125	1	0/8	.....	8/8	.....
			100	1	0/8	.....	0/8	.....
Colchinol methyl ether HCl	2524	Water	250	1	2/8	.....	3/6	.....
			150	1	0/8	.....	5/8	.....

*Expressed as: $\frac{\text{No. of mice dead}}{\text{No. of mice injected}}$.

†Expressed as: $\frac{\text{No. of mice with induced tumor damage}}{\text{No. of mice sacrificed}}$. The tumors in untreated control animals showed no induced damage.

‡Some mice were not sacrificed but were allowed to live for survival determinations.

TABLE 4.—*Approximate values for maximum tolerated dose (MTD) and minimum effective dose (MED) for a single subcutaneous injection of colchicine derivatives in mice bearing Sarcoma 37*

Compound	NCI No.	MTD (μg./gm.)	MED (μg./gm.)	MTD/MED
Colchiceine	1040	150	75	2
Colchicine	1v	3	1.5	2
[N-benzoyltrimethylcolchicinic acid	1038	(*)	(*)	(*)]
N-benzoyltrimethylcolchicinic acid methyl ether	1039	80	40	2
Trimethylcolchicinic acid	1893	600	600	1
Trimethylcolchicinic acid methyl ether d-tartrate	1136	100	2	50
Trimethylcolchicinic acid ethyl ether d-tartrate	2315	100	3	33
O,N-diacetyltrimethylcolchicinic acid	2525	400	200	2
N-acetylcolchinol methyl ether	1895	20	3	7
N-acetylcolchinol	1894	100	4	25
N-acetyliodocolchinol methyl ether	368	400	200	2
N-acetyliodocolchinol	1037	300	50	6
Colchinol methyl ether HCl	2524	250	125	2
Colchinol	1896	500	125	4

*No deaths or induced damage observed at the highest dose given (see table 2). This compound is listed for comparison with No. 1039 in the line below.

parallel increase in MED from 1.5 to 75. When, in addition to demethylation, the amino group in ring B is deacetylated to trimethylcolchicinic acid (NCI No. 1893), both the MTD and MED rose to 600 μg. per gram. Yet deacetylation without demethylation, in the case of trimethylcolchicinic acid methyl ether d-tartrate (No. 1136), resulted in a compound which had an MTD (100 μg. per gm.) some 30 times greater than colchicine, but an MED (2 μg. per gm.) virtually the same as that for colchicine. The ratio of MTD to MED for this compound was about 50, as compared with a ratio of about 2 for colchicine.

No simple explanation for these results is obvious. Goldberg and coworkers (*13*) tentatively suggested that greater biological activity was associated with greater water solubility. However, the correlation between the biological activity of the compounds we studied and their solubility was not consistent with this hypothesis. For example, the least toxic and least effective derivatives [trimethylcolchicinic acid (No. 1893) and N-benzoyltrimethylcolchicinic acid (No. 1038)] form a water-soluble hydrochloride and sodium salt, respectively, whereas N-benzoyltrimethylcolchinic acid methyl ether (No. 1039) is both more lethal to the host and more effective against tumors than the foregoing two compounds, yet it has little solubility in water. Colchiceine (No. 1040) is also readily soluble as a sodium salt and has both low lethality and low tumor-damaging activity. For the colchinol compounds, as well, solubility in aqueous vehicle did not seem to be a factor determining either potency or toxicity. N-acetylcolchinol methyl ether (No. 1895), one of the two compounds in

this series not soluble in aqueous vehicle, was nevertheless the most toxic of the six. It nevertheless had a higher MTD/MED than any of the other compounds except N-acetylcolchinol (No. 1894). Analogously, colchinol methyl ether (No. 2524) formed a water-soluble hydrochloride, yet it was among the least active. There were thus so many exceptions, that solubility did not provide a good correlation with the biological activity of these compounds.

Tables 2 and 4 indicate that the ether is important in endowing the trimethylcolchicinic acid derivatives with high tumor-damaging potency and high toxicity. Three pairs of compounds are shown in the upper part of table 4; when the methylated compounds (colchicine, N-benzoyltrimethylcolchicinic acid methyl ether, and trimethylcolchicinic acid methyl ether d-tartrate) are compared with their corresponding demethylated derivatives (colchiceine, N-benzoyltrimethylcolchicinic acid and trimethylcolchicinic acid), the methyl ether in each instance had both a lower MED and a lower MTD. While colchicine and colchiceine had similar values for the ratio of MTD to MED, the methylated compounds in the other two pairs had higher ratios.

The effect of the methyl group in the "colchinol" series was different. Comparison of the three pairs of methylated and demethylated derivatives in the bottom half of table 4 shows that the methylated compounds had a *lower* ratio of MTD to MED in all three instances.

A radical change in the basic ring structure, *viz.*, degradation from a seven-membered ring to a six-membered one in the colchinol derivatives, resulted in a series of compounds with some members having a greater relative activity than colchicine itself. Thus, of the six compounds investigated in this group, only N-acetyliodocolchinol methyl ether (No. 368) and colchinol methyl ether (No. 2524) had a ratio of MTD/MED which was not greater than that of colchicine. One compound (N-acetylcolchinol) had a ratio of about 25.

On comparison of the ratio of MTD/MED for the compounds in the colchinol series with that of their trimethylcolchicinic acid analogs, it will be seen that in three out of four instances the colchinol compounds were relatively more potent. For this comparison, data from table 4 are regrouped in table 5, which shows that in only one of these four pairs did the trimethylcolchicinic acid derivative have a greater MTD/MED than its colchinol analog.

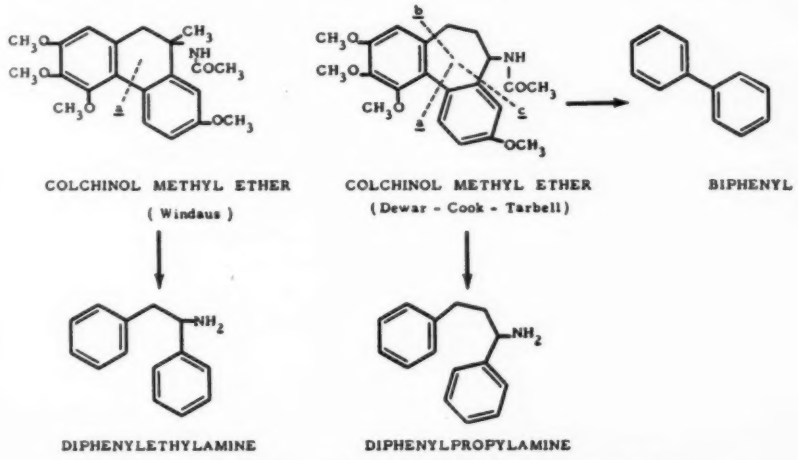
Still more radical variations of the key structure of colchicine have also been investigated for mitosis-arresting activity or tumor-damaging effects. Diphenylethylamines might be considered to be derived from colchinol by opening the B ring of the formula of Windaus in position *a* (text-fig. 4). Many such compounds have been examined by Lettré and Fernholz (*28*) and in this Laboratory (*1*). A high percent were found to have potency as mitotic poisons or as tumor-damaging compounds *in vivo*. As evidence accumulated that the Windaus formula was not correct, and that the B ring was in all likelihood a seven-membered one, the analogous diphenyl-

TABLE 5.—*Effect of aromatization of colchicine C ring on tumor-damaging activity*

Compound	NCI No.	MTD (μg./gm.)	MED (μg./gm.)	MTD/MED
Colchicine	1v	3	1.5	2
N-acetylcolchinol methyl ether	1895	20	3	7
Colchiceine	1040	150	75	2
N-acetylcolchinol	1894	100	4	25
Trimethylcolchicinic acid	1893	600	600	1
Colchinol	1896	500	125	4
Trimethylcolchicinic acid methyl ether d-tartrate	1136	100	2	50
Colchinol methyl ether HCl	2524	250	125	2

propylamines, which would result if the seven-membered B ring were opened, were also examined in our laboratory. A substantial number in this group of compounds also showed activity in damaging Sarcoma 37, *viz.*, of 27 diphenylpropylamines examined to date, 12 consistently produced damage to Sarcoma 37 following a single injection. Lettré and Fernholz (*28*) have reported negative results in arresting mitosis with about ten acetylated diphenylpropylamines. In addition, Skipper, Bell, and Chapman (*29*) obtained negative results with the one diphenylpropylamine they reported.

By an analogous splitting of the B ring at positions *b* and *c* (text-fig. 4) it is possible to obtain a biphenyl compound. In a small series of such compounds, none produced damage in Sarcoma 37 with our technique. In particular, 2′-amino-4′,2,3,4-tetramethoxybiphenyl, which is similar to the biphenyl compound that might be obtained by splitting the B ring of colchinol methyl ether, was inactive at a dose of 500 micrograms per gram



TEXT-FIGURE 4.—Simpler analogs of colchicine, the central ring opened.

of body weight. Dr. E. C. Horning has prepared a series of biphenyl compounds which have been under investigation elsewhere.[6]

Several investigators have compared the biological activity of some of these derivatives with that of colchicine. Most of them have used as criteria the effect on mitosis in different tissues. Brues and Cohen (*30*) employed regenerating liver of the rat. Others used mouse tissues: Branch *et al.* (*12*), gastric mucosa; Goldberg *et al.* (*13*), Sarcoma 180; Fleischmann and Ullyot (*14*), cornea; and Bergner (*15*), spermatogonia. In addition to determining the effects on chick fibroblasts in tissue culture, Lettré (*31*) and Lettré and Fernholz (*28*) also employed survival and weight studies in mice bearing the Ehrlich carcinoma in the form of an ascites tumor. Comparisons with the results of these various investigators is difficult, inasmuch as different derivatives were used as well as different test tissues. However, all these investigations, including our own, demonstrate that considerable modification of the biological activity and of the lethality of colchicine may be obtained with such derivatives.

Although the compounds reported here fall into closely comparable pairs in many instances, the correlations of structure and potency are still based on a relatively small number of compounds. Additional related derivatives are being prepared or are under investigation to assess further the significance of the correlations.

SUMMARY AND CONCLUSIONS

In an investigation employing about 1500 mice bearing Sarcoma 37, fifteen derivatives of colchicine were examined for ability to damage Sarcoma 37 following a single subcutaneous injection. It was found that:

1) Thirteen of the derivatives tested were able to damage the tumor at or below the maximum tolerated single dose.

2) Six of these had a ratio of the maximum tolerated dose to minimum effective dose (MTD/MED) of 4 or greater, whereas the ratio exhibited by colchicine was 2.

3) Three compounds (trimethylcolchicinic acid methyl ether d-tartrate, trimethylcolchicinic acid ethyl ether d-tartrate, and N-acetylcolchinol) had an MTD/MED ratio of 25 or greater.

4) Minor changes in the substituents on the colchicine molecule to form other derivatives of trimethylcolchicinic acid resulted in marked changes in lethality and tumor-damaging potency. Changes in lethality were not always paralleled by equivalent changes in tumor-damaging potency.

5) A more radical change in the colchicine molecule, *viz.*, degradation of the seven-membered C ring to a six-membered aromatic ring, did not abolish potency. Of six such compounds, the MTD/MED for four derivatives was greater than for colchicine.

6) Methylation of the seven-membered C ring in colchicine generally conferred higher relative potency against Sarcoma 37 in comparison with the unmethylated analogs. On the other hand, the methylated compounds

[6] Personal communication from Dr. Horning.

were less potent than the unmethylated when the C ring was a six-membered aromatized one.

7) In three of four analogous pairs, the compounds containing a six-membered aromatic C ring were relatively more potent than their analogs with a seven-membered ring.

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Effect of Podophyllotoxin, *Alpha*-Peltatin and *Beta*-Peltatin on the Cytochrome Oxidase Activity of Sarcoma 37 [1, 2]

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Previous reports (*2–4*) from this Laboratory have shown that three compounds, podophyllotoxin, *alpha*-peltatin and *beta*-peltatin, which were isolated from podophyllin (*5*), had high potency in inducing damage in Sarcoma 37 as well as in a variety of other tumors (*6*). Several derivatives of these compounds were also prepared (*5*) and their potency against tumors was reported (*1*). All three of the compounds had a substantial range between the maximum tolerated dose (30–40 μg. per gm.) and the minimum effective dose (about 2 μg. per gm.) for a single subcutaneous injection. In addition, treatment with alkali converted these substances into diastereoisomers (*5*) of greatly reduced potency (*7*).

These active components of podophyllin, together with their relatively inactive isomers, were therefore considered suitable for mechanism studies to determine the manner in which they induced acute damage in the tumors. Accordingly, enzymatic studies of the effects of podophyllotoxin, *alpha*-peltatin, *beta*-peltatin, and several of their derivatives were undertaken in an effort to determine whether any specific metabolic processes in tumors, as contrasted with nonmalignant tissues, had been affected. The effect on cytochrome oxidase activity of tissues from treated animals, as well as of tissue preparations treated *in vitro* with various compounds, was investigated.

MATERIALS AND METHODS

The compounds employed in these experiments were isolated from podophyllin by Hartwell and Detty (*5*) or synthesized by Hartwell from the active components of podophyllin. The potency of these compounds in damaging Sarcoma 37 was reported elsewhere (*4, 7*).

[1] Received for publication May 12, 1952.

[2] Presented in part at the annual meeting of the American Association for Cancer Research in 1949 (*1*).

[3] This work was carried out during the tenure of the senior author as a Research Fellow of the National Cancer Institute, on leave from the Indian Research Fund Association, Clinical Research Unit, Tata Memorial Hospital, Bombay, India.

[4] National Institutes of Health, Public Health Service, Federal Security Agency.

CAF_1 hybrid mice bearing six-day-old intramuscular implants of Sarcoma 37 were employed in these experiments. In the case of non-tumor-bearing mice, ZBC (Bittner) backcross mice and ABC (Bittner) mice were used.

The compounds were dissolved in dilute sodium hydroxide or a 50 percent aqueous solution of propylene glycol, or else were dispersed in olive oil. The animals were given a single subcutaneous injection of the compounds, and about four were sacrificed at intervals of from 1 to 120 hours after injection. Tumors were observed grossly and microscopically for evidence of induced damage. The techniques employed in the animal experiments have been described in more detail elsewhere (*4*). The tumors, freed from necrotic, connective or muscle tissue or other tissues, were pooled, passed through a sieved press, homogenized in a glass homogenizer, and examined for cytochrome oxidase activity. At least one group of untreated mice for each 24-hour interval was examined in the same way and served as controls. All determinations were performed in duplicate. The ranges of average control values for the cytochrome oxidase activities of the different tissues are given in footnotes in the tables.

In vitro experiments were performed by adding enough of either *alpha*- or *beta*-peltatin to give the desired concentration in the tissue homogenate from untreated mice. The preparations were then incubated for periods of 1 to 3 hours and their cytochrome oxidase activity determined. Tissue homogenates incubated with equivalent amounts of vehicle (dilute aqueous alkali or 50 percent propylene glycol) served as controls.

The cytochrome oxidase activity was determined in the usual fashion with 0.1 to 0.2 ml. of a homogenate of one part wet tissue and nine parts distilled water. The substrate employed was essentially the same as was employed by Schneider and Potter (*8*) (0.3 ml. of 0.114 M ascorbic acid, neutralized to *p*H 7.0; 1.0 ml. of 4×10^{-4} M cytochrome c, Sigma; 0.3 ml. of 4×10^{-3} $AlCl_3$; 1.0 ml. of 0.1 M $NaH_2PO_4 \cdot NaOH$ buffer, *p*H 7.4; distilled water to make a total volume of 3 ml.). 0.1 ml. of a 10 percent NaOH solution was placed in the center cup to absorb liberated CO_2. Corrections for autoxidation were obtained by measuring oxygen uptake in a similar vessel containing the same substrate but no tissue. Values for autoxidation in the vicinity of 35 cu. mm. O_2 per hour were obtained by this method.

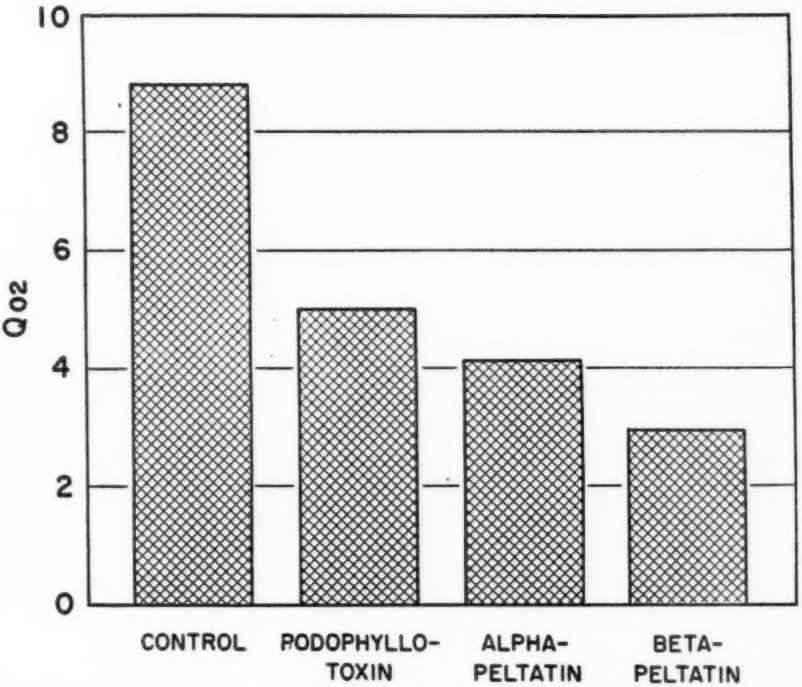
Q_{O_2} values obtained by us under these conditions were considerably lower than those reported by Schneider and Potter, but comparable to the values reported by others using methods other than that of Schneider and Potter. In a few instances the experiments were repeated using a 1 to 100 dilution of tissue according to Schneider's and Potter's method. Values similar to those reported by these authors [5] were obtained, and the changes in cytochrome oxidase activity following treatment with the chemical agents were similar to the ones reported in this paper with a 1 to 10 tissue homogenate.

[5] The tissue dilution method, with extrapolation to zero concentration, was used in determining autoxidation corrections in these determinations. Values for autoxidation in the vicinity of 40 cu. mm. O_2 per hour were obtained. These are similar values to those reported by Schneider and Potter (*8*).

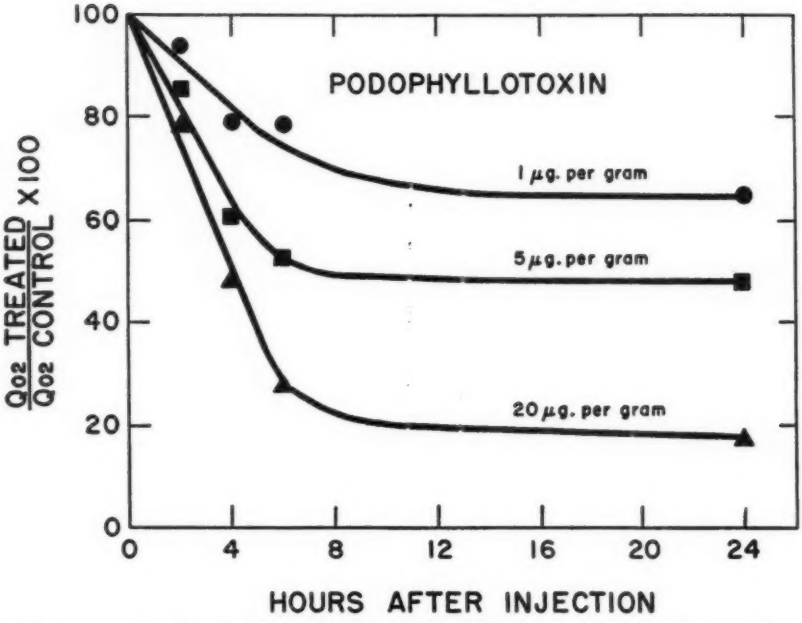
RESULTS

Effect of Alpha-Peltatin, Beta-Peltatin and Podophyllotoxin on the Cytochrome Oxidase Activity of Sarcoma 37

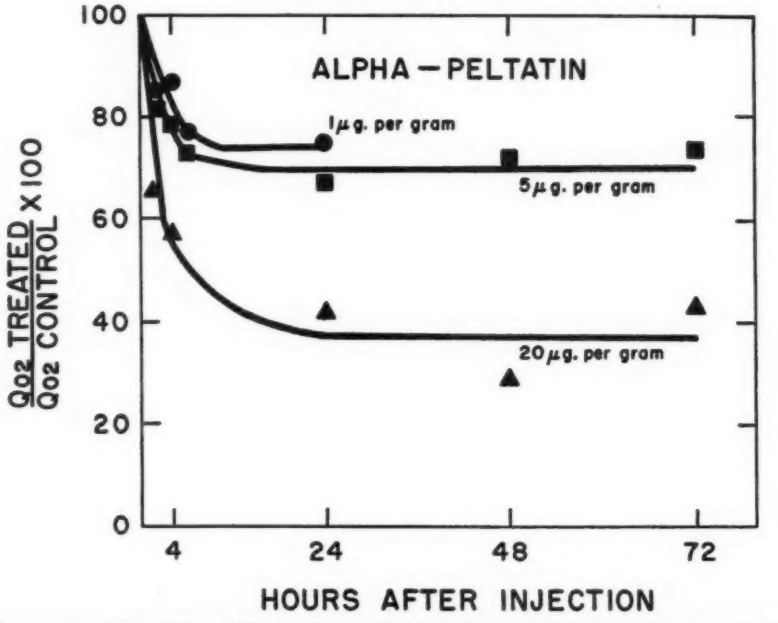
Text-figures 1–4 summarize the changes obtained in the cytochrome oxidase activity of tumor tissue homogenates from animals bearing Sarcoma 37 following a single subcutaneous injection. Text-figure 1 shows the reduction in Q_{O_2} in homogenates from animals killed 24 hours after a single injection of 5 μg. per gm. of podophyllotoxin, or *alpha-* or *beta-*peltatin. Reduction in Q_{O_2} to about one-half or less of the value obtained for homogenates from untreated control mice occurred. Text-figures 2–4 show the effects of 1, 5, or 20 μg. per gm. doses at various time intervals, up to 72 hours, on the cytochrome oxidase activity of Sarcoma 37 homogenates. The lowest dose was about one-half the minimum tumor-damaging dose (MED), the intermediate dose about twice the MED, and the highest dose about one-half to two-thirds the maximum tolerated dose (MTD) (*4*). In all the experiments the reduction in oxygen consumption of the homogenates was more marked with higher doses. For doses greater than the MED the most rapid reduction occurred during the first six or eight hours, at a time when gross or miscroscopic evidence of damage was not



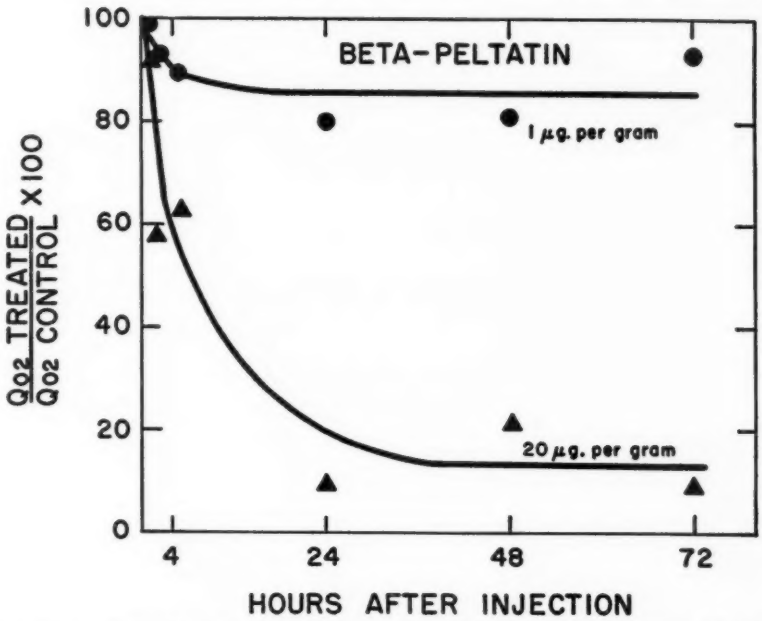
Text-figure 1.—Effect of podophyllotoxin, *alpha*-peltatin and *beta*-peltatin on cytochrome oxidase activity (in cu. mm. O_2/hr./mg. wet tissue) of Sarcoma 37, 24 hours after a single subcutaneous injection of a dose of 5 μg./gm.



TEXT-FIGURE 2.—Effect of 3 dose levels of podophyllotoxin on the cytochrome oxidase activity of Sarcoma 37.



TEXT-FIGURE 3.—Effect of 3 dose levels of *alpha*-peltatin on the cytochrome oxidase activity of Sarcoma 37.



TEXT-FIGURE 4.—Effect of 2 dose levels of *beta*-peltatin on the cytochrome oxidase activity of Sarcoma 37.

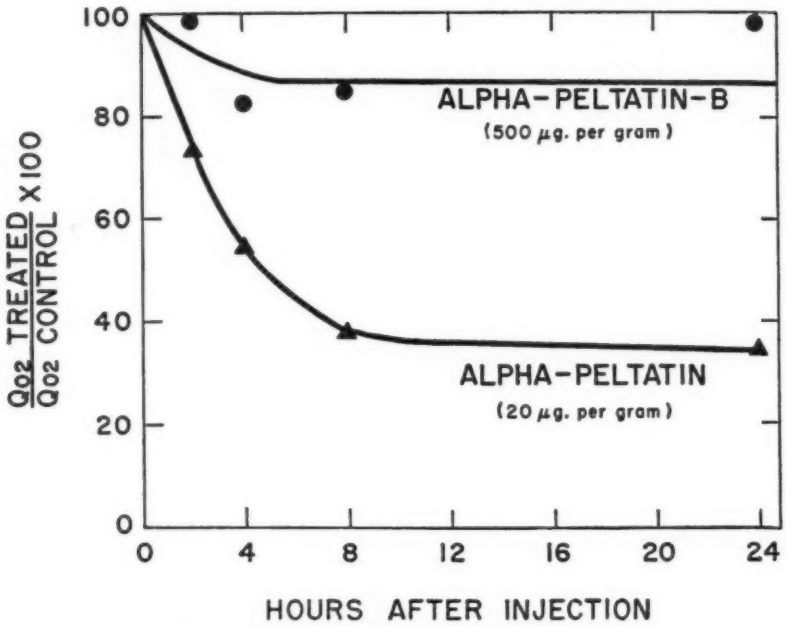
marked (*4*). In 24 hours, in most instances, the rate of oxygen uptake had leveled off to a value less than 50 percent of that of untreated controls. In some instances the Q_{O_2} values were less than 20 percent of those obtained with tumor tissue from control animals. For doses (1 μg. per gm.) below the MED, reduction in enzyme activity was relatively small; any significance attached to these values or the sequence of values would be questionable.

EFFECT OF DERIVATIVES OF PODOPHYLLOTOXIN, ALPHA-PELTATIN AND BETA-PELTATIN ON CYTOCHROME OXIDASE ACTIVITY OF SARCOMA 37

Since Hartwell and Detty (*5*) had shown that these three components of podophyllin could readily be converted into diastereoisomers by inversion of the asymmetric carbon atom adjacent to the carbonyl group, and since it was demonstrated (*7*) that some of these isomers had greatly reduced activity even at doses several hundred times greater than the naturally occurring products, the effect on cytochrome oxidase activity of tumor tissue after injection of these isomers was next determined. The effects of the acetates of podophyllotoxin and of *alpha*-peltatin were also investigated.

Text-figure 5 compares the effect of a 20 μg. per gm. dose of *alpha*-peltatin with that of a 500 μg. per gm. dose of *alpha*-peltatin-B, its diastereoisomer; this huge dose of the isomer produced no gross or irreversible histologic damage in the tumor. The cytochrome oxidase activity of

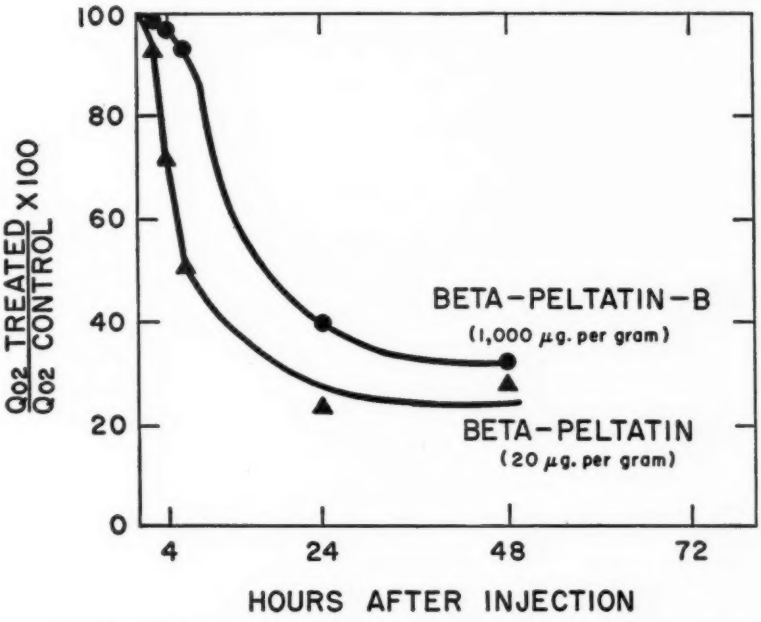
tumor tissue from animals treated with the naturally occurring *alpha* compound dropped rapidly in the first eight hours after injection to a level of less than 40 percent of that of the controls. Its less active diastereoisomer, *alpha*-peltatin-B, on the other hand, caused only a slight drop in cytochrome oxidase activity. The values never fell below 80 percent of those obtained with tumor tissue from untreated controls.



TEXT-FIGURE 5.—Effect of *alpha*-peltatin and its diastereoisomer (*alpha*-peltatin-B) on cytochrome oxidase activity of Sarcoma 37.

The diastereoisomer of the *beta* compound (*beta*-peltatin-B) on the other hand produced gross and histologic damage in Sarcoma 37 after a dose of 1000 μg. per gm. Here, as shown in text-figure 6, a drop in cytochrome oxidase activity of tumor homogenates occurred which paralleled that obtained with 20 μg. per gm. of the naturally occurring *beta*-peltatin. Within 24 hours, an activity of less than 40 percent of the controls was observed for both isomers.

When the acetates of either *alpha*-peltatin or of podophyllotoxin were injected into mice bearing Sarcoma 37 at dose levels of 1000 μg. per gm., neither gross damage nor microscopic evidence of pyknosis was observed (*7*). The comparative effects of these acetates and of their parent compounds, given at much lower doses, on cytochrome oxidase activity are given in text-figures 7 and 8. While both podophyllotoxin and *alpha*-peltatin produced a decrease in enzyme activity to less than 40 percent of the control values, the much higher doses of their respective acetates had much smaller effects.



TEXT-FIGURE 6.—Effect of *beta*-peltatin and its diastereoisomer (*beta*-peltatin-B) on cytochrome oxidase activity of Sarcoma 37.

EFFECT OF THE PELTATINS ON CYTOCHROME OXIDASE ACTIVITY OF NORMAL TISSUES

The effect of these drugs on the cytochrome oxidase activity of normal tissues of mice bearing Sarcoma 37 was compared with the changes produced in tumor tissue. Liver, kidney, and spleen as well as tumor tissue were homogenized, and the cytochrome oxidase activity determined. Table 1 illustrates the results obtained at various time intervals after an injection of 20 μg. per gm. of *alpha*-peltatin. In the case of the tumor

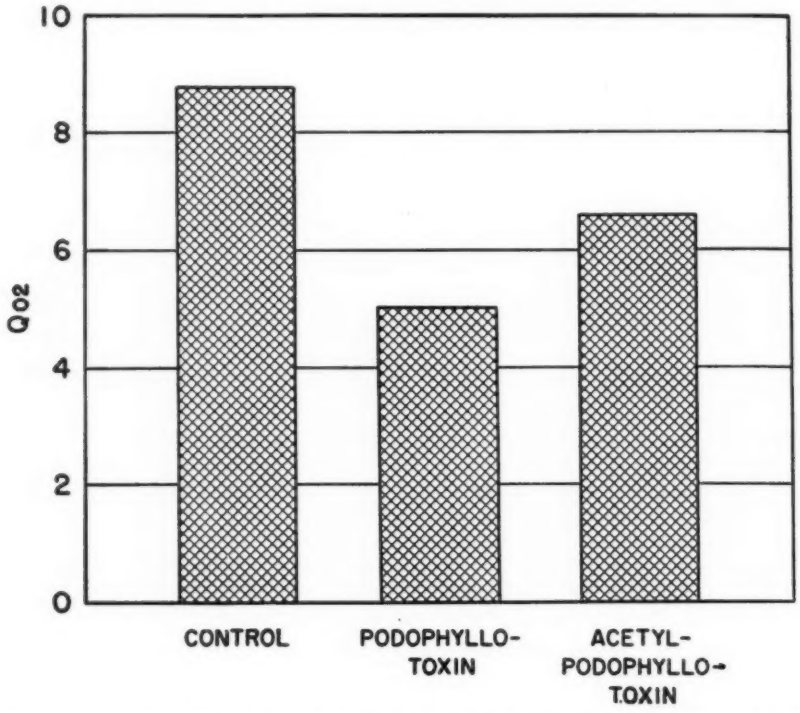
TABLE 1.—In vivo *effect of* alpha-*peltatin on cytochrome oxidase activity in mice bearing Sarcoma 37*

Time after injection* (hours)	Residual activity†			
	Tumor (percent)	Liver (percent)	Kidney (percent)	Spleen (percent)
2	66	82	96	92
4	57	82	96	89
24	42	84	100	95
48	30	94	91	96
72	44	93	69	87
96	....	85	64	88

* 20 μg./gm. of *alpha*-peltatin injected subcutaneously.

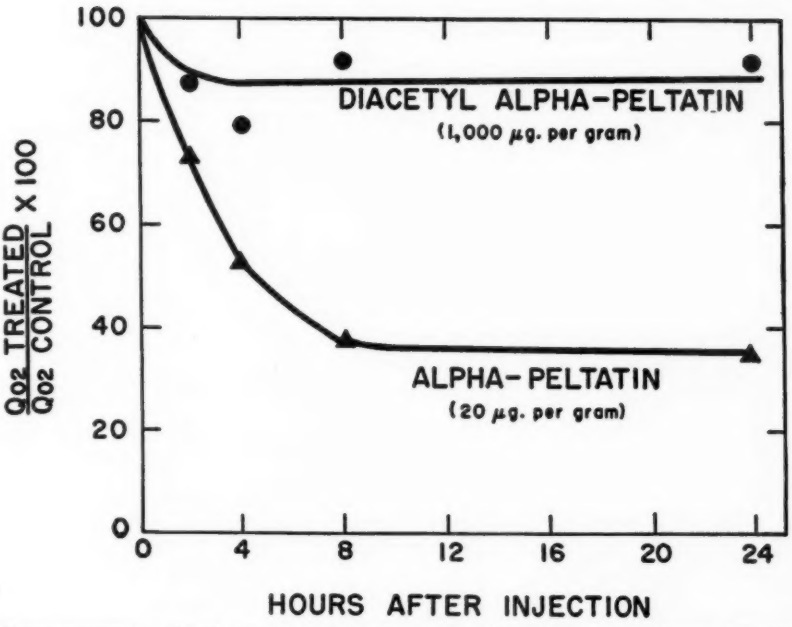
† $\frac{Q_{O_2}\ \text{treated}}{Q_{O_2}\ \text{controls}} \times 100$. Q_{O_2} values (cu. mm. O_2 per mg. wet tissue per hr.) from untreated mice: Sarcoma 37 — 7.9–8.4; kidney — 23.1–25.5; liver — 18.4–20.5; spleen — 9.2–9.8.

tissue homogenates, the enzyme activity dropped rapidly and reached values as low as 30 percent of that of the tumor tissue controls. The values of the other tissues usually dropped only slightly. Generally, over three-fourths of the enzyme activity remained at any time.



TEXT-FIGURE 7.—Effect of podophyllotoxin (5 μg./gm.) and its acetate (1000 μg./gm.) on cytochrome oxidase activity of Sarcoma 37.

Even when supralethal doses of 100 μg. per gm. of *alpha-* or *beta-*peltatin were injected into non-tumor-bearing mice, the reduction of cytochrome oxidase activity of the tissues from treated animals as compared with those from untreated ones was not as marked as that observed with tumor tissue. Tables 2 and 3 summarize these results. Generally, 70 percent or more of the enzyme activity remained.



TEXT-FIGURE 8.—Effect of *alpha*-peltatin and its diacetate on cytochrome oxidase activity of Sarcoma 37.

TABLE 2.—In vivo *effect of a lethal dose of* beta-*peltatin on cytochrome oxidase activity in mice without tumors*

Time after injection* (hours)	Residual activity†			
	Liver (percent)	Kidney (percent)	Spleen (percent)	Testis (percent)
1	91	80	90	99
3	87	75	93	83
5	87	84	97	92
24	95	90	51	69

* 100 μg./gm. of *beta*-peltatin injected subcutaneously.

† $\frac{Qo_2 \text{ treated}}{Qo_2 \text{ controls}} \times 100$. Qo_2 values (cu. mm. O_2 per mg. wet tissue per hr.) from untreated mice: liver — 18.1–20.6; kidney — 23.4–25.8; spleen — 11.8–12.2; testis — 10.0–11.2.

TABLE 3.—In vivo *effect of a lethal dose of* alpha-*peltatin on cytochrome oxidase activity in mice without tumors*

Time after injection* (hours)	Residual activity†			
	Liver (*percent*)	Kidney (*percent*)	Spleen (*percent*)	Testis (*percent*)
2	90	84	91	92
4	82	74	88	87
24	77	70	74	....
48	74	70	76	89
120	72	65	83	84

* 100 μg./gm. of *alpha*-peltatin injected subcutaneously.

† $\frac{Qo_2 \text{ treated}}{Qo_2 \text{ controls}} \times 100$. Qo_2 values (cu. mm. O_2 per mg. wet tissue per hr.) for control tissues: liver — 17.9-20.8; kidney — 24.6-25.5; spleen — 12.9-13.7; testis — 10.8-11.8

EFFECT OF THE PELTATINS ON SARCOMA 37, LIVER AND KIDNEY TISSUE IN VITRO

Homogenates of Sarcoma 37, and of liver and kidney from mice bearing six-day-old implants of the tumor were prepared, and *alpha-* or *beta-*peltatin was incorporated in the homogenates in concentrations of 10 to 100 μg. of drug per ml. of homogenate. The homogenates were incubated in a water bath at 37° C. for 1 to 3 hours, and the cytochrome oxidase activity determined. These values were compared with the values from the same homogenates incubated for the same periods of time but without the addition of drug. Tables 4 and 5 summarize the results. In the lowest concentration (10 μg. per ml.), most of the enzyme activity (80 percent or more) was left unimpaired; in the highest concentration (100 μg. per ml.), about two-thirds of the activity remained. Thus the ability of the peltatins to reduce the cytochrome oxidase activity of tumor tissue appeared to be less *in vitro* than *in vivo*. On the other hand, the effect on normal tissues appeared to be about the same both for the *in vivo* and *in vitro* experiments, *i. e.*, only small reductions over that of untreated tissues.

TABLE 4.—In vitro *effect of* alpha-*peltatin on cytochrome oxidase activity*

Tissue	Concentration of *alpha*-peltatin (μg./ml.)	Residual activity* (*percent*)
Sarcoma 37	10	82
	100	61
Liver	10	86
	100	77
Kidney	10	92
	100	80

* $\frac{Qo_2 \text{ treated}}{Qo_2 \text{ controls}} \times 100$ after incubation for 3 hrs. at 37° C. Qo_2 values (cu. mm. O_2 per mg. dry tissue per hr.) for control tissue: Sarcoma 37 — 40.1; liver — 78.0; kidney — 92.3.

TABLE 5.—In vitro *effect of* beta-*peltatin on cytochrome oxidase activity*

Tissue	Time of incubation (*hours*)	Residual activity* at various drug concentrations	
		20 *μg./ml.*	100 *μg./ml.*
Sarcoma 37.....	1	92	86
	3	79	68
Liver.....	1	87	82
	3	70	89

* $\frac{Q_{O_2}\text{ treated}}{Q_{O_2}\text{ controls}} \times 100$ incubated at 37° C. Q_{O_2} values (cu. mm. O_2 per mg. dried tissue per hr.) from homogenates incubated with vehicle but without drug: Sarcoma 37 — 41.7–42.6; liver — 73.6–74.4.

The incubation of tissues with the peltatins could not be carried out for periods longer than three hours. A rapid falling off of enzyme activity even in the untreated homogenates occurred after this time interval.

EFFECT OF AUTOLYSIS ON THE CYTOCHROME OXIDASE ACTIVITY OF SARCOMA 37

Some investigators (*9*, *10*) have attributed hemorrhage and necrosis in tumors to the ability of certain substances to damage newly formed capillaries, or to a marked reduction in the capillary bed of the tumors. A simple autolysis experiment was selected to determine the effect of such secondary factors as circulatory stasis on the cytochrome oxidase activity. Mice bearing Sarcoma 37 were killed by cervical dislocation and the intact carcasses incubated at 37° C. Tumor and liver tissue were obtained from incubated intact carcasses at various time intervals after death (2, 4, 6, and 24 hours) and the cytochrome oxidase activity determined; control tissues were taken from mice immediately after they were killed. Table 6 summarizes the results. After 2, 4, and 6 hours incubation, the enzyme activity was virtually the same as in tissues from control animals. After 24 hours of incubation, autolysis of carcasses, and especially of liver and tumor, was advanced, and the enzyme activity in these tissues was not measurable.

TABLE 6.—*Cytochrome oxidase activity during autolysis*

Time* (*hours*)	Percent residual activity†	
	Tumor	Liver
2	97	98
4	97	98
6	91	93
24	0	0

*Tumor-bearing animals killed and incubated intact at 37° C.

† $\frac{Q_{O_2}\text{ test}}{Q_{O_2}\text{ control}} \times 100$. Q_{O_2} (cu. mm. O_2 per mg. wet tissue per hr.) for control tissues: Sarcoma 37 —8. 6; liver — 19.6.

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In contrast with the slight changes in enzyme activity, sections of tumor tissue examined during the first 6 hours showed microscopic changes similar to those obtained at similar time intervals after drug administration (*4*). Retraction of cytoplasm and beginning of pyknosis of cell nuclei were prominent.

DISCUSSION

The data presented here demonstrate that subcutaneous injection of these tumor-damaging compounds produced a rapid and marked reduction in the cytochrome oxidase activity of Sarcoma 37. In the case of all the potent compounds, the most rapid fall in enzyme activity occurred during the first six to eight hours after injection. Hemorrhage in the tumor was evident only toward the end of this time interval; although microscopic evidence of cellular damage was seen during this early period, frank induced necrosis was not common. Extensive gross and microscopic damage in the tumors was observed only later, after the cytochrome oxidase activity had virtually leveled off. The percentage drop in enzyme activity was less marked in liver, kidney, spleen, and testis of the tumor-bearing mice, and of non-tumor-bearing mice that had been given even a supralethal dose.

Incubation *in vitro* of tumor tissue homogenates, as well as homogenates of kidney and liver, with *alpha*- or *beta*-peltatin produced smaller reductions in the cytochrome oxidase activity. These data are in accord with the results reported by Kelly *et al.* (*11*) from this Laboratory, that podophyllotoxin is rapidly converted in the body into a form no longer lethal to the chick embryo and incapable of producing damage to tumors; whereas incubation *in vitro* of tissue homogenates with this drug had a much smaller effect on egg toxicity or tumor-damaging potency.

Autolysis of intact carcasses of tumor-bearing mice resulted in histologic changes in the tumors similar to those observed at comparable time intervals after injection of tumor-damaging doses of podophyllotoxin, of *alpha*- or of *beta*-peltatin. Nevertheless, autolysis did not produce significant changes in the cytochrome oxidase activity of the tumor tissue during these periods. Such observations suggest that anoxia following blood stasis or capillary damage may not be the only mechanism by which necrosis is produced in tumors by drugs. Rather, the data presented here suggest that necrosis could be a direct consequence of a cytotoxic effect on tumor tissue.

Boyland and Boyland (*12*) have reported reduction in respiration of tumor tissue slices following injection of colchicine. We have reported briefly (*13*) similar marked reduction in cytochrome oxidase activity of Sarcoma 37 following injection of colchicine and of some of its derivatives. The similarity in pharmacologic action of colchicine and the compounds derived from podophyllin is thus paralleled by their similar action on

cytochrome oxidase activity. Following our preliminary reports (*1*), Miller and coworkers (*14*) were able to confirm these results by reporting a reduction in respiration of tumor slices following the injection of podophyllotoxin.

On the basis of calculations from available data, Greenstein (*15*) has pointed out that tumor tissue appears to have a limited oxidative capacity via the cytochrome system, in contrast with some normal tissues which have a considerable reserve. He has also suggested that tumors may "function to the limit of their available catalytic systems whereas normal tissues have the advantage of a considerable reserve of such systems." From such considerations, the data presented here raise the question as to whether the further marked reduction in oxidative capacity represented by the large decreases in cytochrome oxidase activity of tumor tissue following treatment with tumor-damaging compounds has produced a functional deficiency in the tumors. In the case of normal tissues, similar absolute reductions in enzyme activity, such as were observed here, could still leave a sufficient excess of enzyme (in relationship to the cytochrome c in the tissues) to be able to carry out normal oxidative functions.

These considerations are to some extent borne out by the data of Boyland and Boyland (*12*) and of Miller, Davison, and Smith (*14*) cited earlier. These investigators showed that the oxygen uptake of slices after treatment with colchicine or podophyllotoxin was greatly reduced in tumor tissue slices but little changed in the case of liver slices. This parallels our findings on the reduction of the cytochrome oxidase activity of such tissues in relationship to their oxidative capacities. Furthermore, such evidence as is available from the work of these investigators indicates that anaerobic glycolysis of these tissues is reduced in animals following treatment with these compounds, and that additional energy is probably not furnished through such a pathway.

It should be emphasized however, that the data presented here do not as yet justify a conclusion that there is necessarily a cause and effect relationship between the enzymatic changes and the subsequent necrosis observed. Further experimental data are necessary to establish such a relationship. Parallel investigations of enzyme systems other than the cytochrome need to be made. We have already reported briefly (*13*) the effects of some of these compounds, under similar experimental conditions, on dehydropeptidase and aldolase activities. During the period when the cytochrome oxidase activity dropped most sharply, there were no significant changes in the dehydropeptidase or aldolase activities. Only after necrosis and disintegration in the tumors was marked, and after the cytochrome oxidase activity had leveled off (24 to 48 hours after injection of the compounds), did the activities of these enzymes fall to low levels. Details of these experiments are being prepared for publication.

SUMMARY

The effect of a single injection of tumor-damaging compounds in mice bearing Sarcoma 37 on the cytochrome oxidase activity of homogenates of tumor and normal tissues was investigated. The following results were obtained:

1) A marked drop in cytochrome oxidase activity in tumor tissue was observed following a single injection of effective doses of podophyllotoxin, or of *alpha*- or *beta*-peltatin, naturally occurring components of podophyllin. Enzyme activities were observed which were as low as 20 percent of the values obtained in tumor tissue from untreated animals.

2) The drop in cytochrome oxidase activity was most rapid during the first 8 hours after injection of the drugs, and before any frank necrosis could be observed. By 24 hours, the drop had leveled off. At this time necrosis was extensive throughout the tumor.

3) Doses of podophyllotoxin or of the peltatins or of any of their derivatives, that produced no damage to the tumors, did not produce appreciable change in the cytochrome oxidase activity.

4) The cytochrome oxidase activity of liver, kidney, spleen, and testis from mice receiving even a supralethal dose of the active compounds showed relatively small decrease. Usually over seventy percent of the activity of these tissues was retained.

5) Incubation *in vitro* of homogenates of tumor, kidney, or liver with concentrations of *alpha*- or *beta*-peltatin of up to 100 μg. per ml. generally produced smaller depressions of the cytochrome oxidase activity than did injections of the same compounds *in vivo*.

6) Autolysis of intact carcasses of tumor-bearing mice for periods up to 6 hours at 37° C. produced histologic changes similar to those observed after similar time intervals following the injection of a tumor-damaging compound, but did not produce a significant decrease in the cytochrome oxidase activity of the tumor tissue.

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A Transplantable Ovarian Papillary Adenocarcinoma of the Rat with Ascites Implants in the Ovary[1]

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The occurrence of spontaneous ovarian tumors in laboratory rodents is not frequent as far as can be ascertained from the literature. Slye *et al.* (*1*) found, among 22,000 mice, 44 with spontaneous ovarian tumors. Most of the tumors were benign papillary adenomas, while four were primary malignant ovarian tumors. The authors also mentioned, in a review of the literature, eight reports of tumors arising in the ovaries of mice. Other investigators reported spontaneous granulosa-cell tumors of the ovaries in mice (*2, 3*) and rarely, ovarian teratomas (*4*). At the National Cancer Institute, three malignant teratomas of the ovaries of spontaneous origin have been found in strain C3H adult female mice.[5] Ovarian tumors are rare in mice and rats. Bullock *et al.* (*5*), and Curtis *et al.* (*6*) found six spontaneously occurring ovarian tumors, equally divided between carcinomas and sarcomas, in 14,638 female rats of five different strains. The rats were over 19 months of age, and the tumors were distributed as follows: Fischer strain—one carcinoma and two sarcomas; August strain—one carcinoma and one sarcoma; Copenhagen strain—one adenocarcinoma. No ovarian tumors were found in rats of the Marshall and Zimmerman strains. The three ovarian carcinomas were papillary cystadenocarcinomas. Ratcliffe (*7*) reported three ovarian carcinomas in 295 tumor-bearing female rats of the Wistar strain. The 295 rats with spontaneous neoplasms represented 6.7 percent of the total female population of the colony. Animals having ovarian tumors averaged approximately 17 months of age.

Recently, Iglesias *et al.* (*8*) reported a transplantable functional ovarian tumor in a strain AXC rat. The tumor was of the granulosa-cell type mixed with large lutein cells.

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Extensive work has been done on ovarian tumors that were induced in mice by irradiation (*9–12*) and in mice and rats by intrasplenic ovarian grafts (*13–18*), and many of these tumors were transplanted (*19–21*). The experimentally induced ovarian tumors were nonfunctional tubular adenomas and functional granulosa-cell tumors and luteomas. The benign and malignant papillary ovarian neoplasms, which are the commonest ovarian tumors of women, have not been thoroughly investigated in experimental animals. With the exception of a short gross and microscopic description by Curtis *et al.* (*6*) of three papillary cystadenocarcinomas in rats, neither detailed studies nor transplantation of this type of ovarian tumor have been made.

The occurrence of a mucinous papillary adenocarcinoma in the ovary of a 9-month-old Osborne-Mendel rat is reported here. The tumor has been successfully transplanted by subcutaneous, intraperitoneal, and intracranial routes. At the present time, the tumor is in its 25th transplant generation, and it is carried on in two lines: 1) intraperitoneally by tumor ascites fluid injections, and 2) subcutaneously by tumor-tissue transplants.

The tumor arose apparently spontaneously in an untreated animal. The mother of this tumor-bearing rat, however, had been fed 2-acetylaminofluorine during lactation. This paper deals with a description of the original tumor, its histopathologic characteristics, certain transplantation experiments with it, and the production of large ovarian tumors by it following intraperitoneal inoculation.

ORIGINAL TUMOR

Gross description.—At autopsy the peritoneal cavity of the rat was found to be filled with bloody fluid. A tumor mass measuring 5.2 × 4.3 × 3.6 centimeters, with a round and slightly lobulated smooth surface, involved the right ovary and oviduct (fig. 1). It was pinkish-white in color, and soft in consistency. In cross section, the tumor was found to be solid, with large necrotic and hemorrhagic zones in the central part (fig. 2). The left ovary was normal in size and shape and appeared grossly to be free of tumor. A soft mass of polypoid growth, 0.5 centimeters in thickness and grayish-red in color, was spread across the visceral and parietal peritoneal wall, the omentum, and the mesentery. Metastasis had occurred to the abdominal and mediastinal lymph nodes, the liver, and both lungs. On the surface of both lungs were grayish-red nodules of pinhead size and slightly larger. Three of these were seen on the right lung and four on the left. Two nodules of similar size and color were found in the right lobe of the liver.

Histologic methods.—The tissues of the original tumor and the transplants were fixed in Zenker's acetic acid fluid, 10 percent formalin, and Carnoy's fluid, and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin, and by the Masson trichrome technique. Best's carmine method for glycogen, the periodic acid-Schiff reaction (PAS), Mayer's mucicarmine, and Wilder's silver impregnation

method for reticulum were also employed. Smears from the ascitic fluid were stained by hematoxylin and eosin or by the Papanicolaou stain.

Microscopic description.—The tumor showed a variable pattern. Some areas showed solid groups of cells, some had an alveolar or cordlike structure, and others showed a definite adenomatous and papillary arrangement (fig. 3). Very often all these patterns could be seen in the same microscopic field.

The tumor was surrounded by a thick vascularized connective tissue capsule, which at the pedicle penetrated part of the tumor and divided it. This connective tissue showed a lymphocytic- and granulocytic-cell infiltration with focal hemorrhages. The stroma of the tumor was formed by a fine fibrillar network that was more prominent at the periphery of the tumor where a solid pattern was often found. A remarkable feature of the tumor was the formation of an acidophilic, homogeneous material. This material seemed to be a secretory substance produced by tumor cells which occasionally revealed the presence of cytoplasmic acidophilic granules or round or oval-shaped masses within vacuoles. Moreover, some early papillary formations consisted of goblet-type cells (fig. 4). The tumor cells appeared to be surrounded by this substance, and the fibrillar stroma was often obscured by it. Occasionally, the homogeneous material was so extensive that the glandular formations or individual cells were isolated in it. This material contained no glycogen. With the mucicarmine stain the homogeneous material stained pinkish-red in some areas, and was metachromatic (toluidine blue). This substance in both intracellular and extracellular locations was reddish in color in preparations with periodic acid-Schiff reaction. It will be designated as "mucoid" substance until further histochemical studies may determine its nature more exactly.

Variation in size and shape of the tumor cells seemed to be correlated with variation in the supporting stroma. In the denser portions of the tumor with abundant stroma, the cells were usually small with a round nucleus, scant and ill-defined cytoplasm, and a coarse, stippled pattern of the chromatin. In the areas with loose stroma, the cells were larger and contained the acidophilic homogeneous material described above. In papillary formations, they were cuboidal or flat, and arranged in a single row. Hyperchromatic and distorted nuclei, and cells in mitotic division, were also seen. Large necrotic zones showed eosinophilic "ghost" cells or granular debris.

In a well vascularized area of the tumor near its capsule, several foci of multinucleated syncytial cells with hyperchromatic nuclei and deeply stained cytoplasm were found. These cells were located adjacent to or within blood sinusoids, and close to them cuboidal cells with faintly stained cytoplasm were seen. Other areas consisted of rather solid portions occasionally forming glandlike spaces lined by large epithelioid cells.

The histology of the tumor closely resembled that of a papillary adenocarcinoma of the ovary, with conspicuous formation of a mucoid material

probably secreted by the tumor cells. The tumor implants in the peritoneal cavity, as well as the tumor metastases in the lymph nodes, liver, and lungs showed the same microscopic characteristics as the primary tumor. Tumor emboli have been found microscopically in the branches of the pulmonary artery in the lungs.

The left ovary was examined in serial section, and although it appeared to be normal on gross examination, microscopically a very small polypoid growth with a narrow pedicle was seen arising from its surface near the hilus (fig. 5). The growth was composed of nests of clear cells with scant granular cytoplasm, surrounded by a few spindle-shaped cells and a very delicate reticulum. The arrangement of the cells was adenomatous or tubular, with formations in some areas reminiscent of rudimentary gonadal structures (anovulatory ovarian follicles and male seminiferous tubules) (figs. 6 and 8). Some glands or tubules which were dilated showed infolding or invaginations, with a tendency toward papillary formation (fig. 7). On the surface of the polypoid growth, the so-called "germinal" epithelium appeared to be invaginated and to be in direct continuity with the adenopapillary formations (fig. 5). The scant stroma around adenomatous or follicle-like formations displayed the characteristics of ovarian stroma. The structure of the polypoid growth showed clearly that it derived from the left ovary in which it arose, and probably from its "germinal" epithelium. No metastases from the malignant neopolasm of the right ovary were found in the left ovary.

TRANSPLANTATION EXPERIMENTS

Transplants.—Tissue from the original tumor was immediately transplanted into female rats of the same strain, subcutaneously by grafts, and intraperitoneally by injecting the ascitic fluid.

Transplantation experiments were carried out by serial passages in female rats of the same strain (Osborne-Mendel). A few rats of other strains (Sprague-Dawley, Marshall-520, and Wistar) were also used. The tumor was transplanted only occasionally to male rats. It was transplanted subcutaneously, intraperitoneally, and intracranially. For subcutaneous and some intraperitoneal transplants, the trocar method of implanting tumor tissue was used. Ascitic fluid containing tumor cells was injected intraperitoneally in most of the peritoneal transplants. Three newly born Sprague-Dawley rats were injected intracranially with 0.5 cc. of ascitic fluid. Following are the results of the twelve first-transplant generations.

Results of the transplantation experiments.—The tumor was transplanted subcutaneously into 31 Osborne-Mendel rats (29 females and 2 males), and grew successfully in 27 females and in both males (93.5% of the animals). This was the strain of rat in which the tumor originated. Intraperitoneal transplants were made successfully in 31 of 35 female rats (88.6%). These intraperitoneal transplants were tissue transplants in 9 of the rats (all successful), and ascitic fluid transplants in 26 of the rats

(22, or 84.6% successful). The ages of the hosts varied from 36 to 223 days at the time the transplants were made.

Growth of transplants in the Osborne-Mendel rats.—Successful transplantation was obtained at all ages, without any significant differences in the rate of growth. A significant difference was observed in the survival of tumor-bearing rats after transplantation depending upon the site of transplantation. In those with subcutaneous transplants, the average time of death was 68 days after transplantation. The subcutaneous transplant appeared as a small palpable nodule about two weeks after transplantation, reaching a size of 3 to 4 centimeters within four or five weeks. It killed the animal nine or ten weeks after transplantation. Metastases to regional lymph nodes or to the lungs were occasionally seen.

In rats with peritoneal transplants, the average survival time was 40 days. In these animals, swelling of the abdomen due to ascites and tumor was noticed at about two weeks after transplantation. The swelling increased, and the animal died in about six weeks. At autopsy, usually a large soft polypoid mass was found to involve the omentum, with papillomatous nodules spreading throughout the mesentery and peritoneal wall. The polypoid mass was implanted on the capsules of the liver and spleen, and metastases to the abdominal and mediastinal lymph nodes, pancreas, liver, spleen, and lungs often developed.

The selective localization in the ovary of the tumor tissue growing after intraperitoneal transplants was strikingly consistent. The ovaries were involved in 83.9 percent of 31 female rats receiving intraperitoneal transplants that grew. Bilateral implantation in the ovaries was found in 22 rats, and in 4 animals only one ovary appeared to be involved. The size of the ovarian tumors varied from a few millimeters to 3 or 4 centimeters in their largest dimensions, and in one instance a tumor (fig. 9) approximately the same size and form as the original one developed. Others were not seen grossly, but in microscopic sections of the ovaries, early growths in the saccular space or in the corpus luteum were found (figs. 13, 14, and 15).

Transplantation in newborn and adult rats of other strains (Marshall-520, Wistar, and Sprague-Dawley).—Intraperitoneal transplants were successful in 5 of 6 adult female Marshall-520 rats, with bilateral ovarian implantation resulting in two of them. Of the intraperitoneal transplants in four adult female Wistar rats, the tumor grew in one rat, and only in the peritoneal cavity. Seven 1-day-old Sprague-Dawley rats received transplants with ascitic fluid—two female and two male rats intraperitoneally, and three male rats intracranially. All of these animals grew normally, and developed tumors. They were killed within 39 to 48 days after transplantation. Both female rats developed bilateral ovarian tumors from the implants. From the intracranial implants in rats, tumors proliferated on the meninges of the brain (fig. 10), with characteristic papillary formation. In one of these, the neoplasm invaded the bones at the base of the skull.

Miscroscopic findings in transplated tumors.—In the early growths on the serosal surfaces (peritoneum and meninges), readily apparent damage to the coeliac lining cells could be seen (*22*). The cells were swollen and showed degenerative changes and necrosis. The exfoliated serosal surface was in places covered by tumor cells, which produced excrescences or large papillomatous masses (fig. 11), sometimes infiltrating the subserosal connective tissue. The tumor infiltration extended sometimes into the parenchyma of organs beneath the visceral serosa. Metastases in the lymph nodes (fig. 12), the abdominal organs, and in the lungs were found microscopically quite often in organs that had appeared to be normal upon gross examination. The omentum became extensively involved in the rat, probably due to a falling of the tumor mass because of gravitational factors, such as occurs in pelvic peritoneal implantation of tumor tissue in man. A serosanguineous effusion was the result of the serosal carcinomatosis.

By careful study of the sections from the tumor transplants, it could be ascertained that the growth in the ovary was an implantation. In early implants, the ovary upon gross examination appeared to be normal. Microscopically, however, small clusters of tumor cells and minute adenopapillary growths could be seen in the space of the ovarian capsule, suggesting that this locus may be a favorable culture medium for implants (figs. 13 and 14). Evidently soon thereafter, the ovary itself was involved. Generally, a corpus luteum was apparently the first to be invaded, and after that the luteal cells were replaced by the adenopapillary growths (fig. 15). In later stages, the tumor completely infiltrated the functional parenchyma, which still maintained the shape of the ovary and of the individual corpora lutea. It is probable that the tumor cells reached the ovary through the small opening in the ovarian sac which is a characteristic structure of the mouse and of the rat (*23*, *24*). This phenomenon of "organotropy" of tumor cells toward the ovary of the host, as in the Krukenberg ovarian tumor, is the subject of another study with this tumor in comparison with other transplantable tumors of rats.

The ascitic fluid.—The ascitic tumor fluid was hemorrhagic, containing a large number of tumor cells that appeared singly or in clusters of two to twelve cells. The cells, varying in size and shape, were round with deeply stained nuclei. The cytoplasm was basophilic in some cells, and neutrophilic in others; and in still others, it was acidophilic with intracytoplasmic vacuoles and secretion droplets.

DISCUSSION

The primary ovarian tumor reported here showed through its serial passages fundamentally the same pattern as the original tumor. There was no important change that could be accounted for by age or strain differences of the hosts, the number of passages, or by the place of transplantation. The giant cells and syncytium-like tissue found in a few foci of the original tumor were not seen in any of the serial passages of the tumor transplants.

The tumor did not show any hormonal-secretory activity. The endometrium of the tumor-bearing rats was always found to be normal. In animals with intraperitoneal transplants of the tumor, the endometrium was found to be in a resting phase, with no hyperplasia of mammary gland tissue. Hypervolemia, which accompanies some granulosa-cell tumors of the mouse, was not found. The vaginal smears of some rats revealed irregularities of endometrial cycle, with tendency toward a permanent state of diestrus that could be accounted for by spontaneous castration due to involvement of the ovaries by the tumor. However, the same type of estrus cycle was observed in some animals having intraperitoneal implants without involvement of the ovaries. The testes of male rats bearing the tumor did not show any changes. Active spermatogenesis with no increase of interstitial cells was found.

It is important to note that the tumor grew very well on the serosal surfaces (peritoneum, meninges, bursa-ovarii), where rich growth of papillary formations was seen. The adenopapillary pattern of the tumor, its secretion of a mucoid substance, and its tendency to papillary serosal implantation, resemble the features of pseudomucinous papillary cystadenomas and papillary adenocarcinomas of the human ovary.

Primary bilateral ovarian growths are not infrequent (*25*). The small polypoid growth found microscopically in the left ovary of the original rat was a second primary histologically benign ovarian neoplasm which apparently derived from the germinal epithelium. It was a so-called tubular adenoma with a tendency toward papillary growth and formation reminiscent of rudimentary gonadal structures. It is worth mentioning that in mice treated with X ray, the altered ovaries (*26*, *27*) and the subsequently developing ovarian tumors (*19*, *28*) showed similar structures, and that embryologic studies indicate that the surface epithelium of the ovary is pluripotent (*29*).

It may be that the malignant tumor of the right ovary originated, like the polypoid growth in the left ovary, in the germinal epithelium and differentiated into tissue components resembling derivatives of the müllerian duct (*30*) with the formation of adenopapillary structures consisting of epithelial cells that secrete a mucoid substance.

SUMMARY

An ovarian papillary adenocarcinoma was found in a 9-month-old Osborne-Mendel rat. The main characteristics of the tumor were its definite adenopapillary pattern, with a conspicuous mucoid substance, its predilection for implanting in the ovary after intraperitoneal transplantation, and its high degree of malignancy. The tumor showed no evidence of hormonal secretory activity. It proved to be readily transplantable both subcutaneously and intraperitoneally to serosal surfaces, and readily transplantable in the same strain of rat with some success in other strains. The tumor is believed to be derived from the germinal epithelium.

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PLATE 38

FIGURE 1.—Original ovarian tumor at the time of autopsy. Tumor implants can be seen in the omentum and mesentery, and small nodules (tumor metastases) appear in the lower lobe of the left lung.

FIGURE 2.—Ovarian tumor and attached uterine horn. ¾ natural size.

FIGURE 3.—Original ovarian tumor, showing the papillary and glandular pattern, with strands of mucoid-hyaline material. × 100

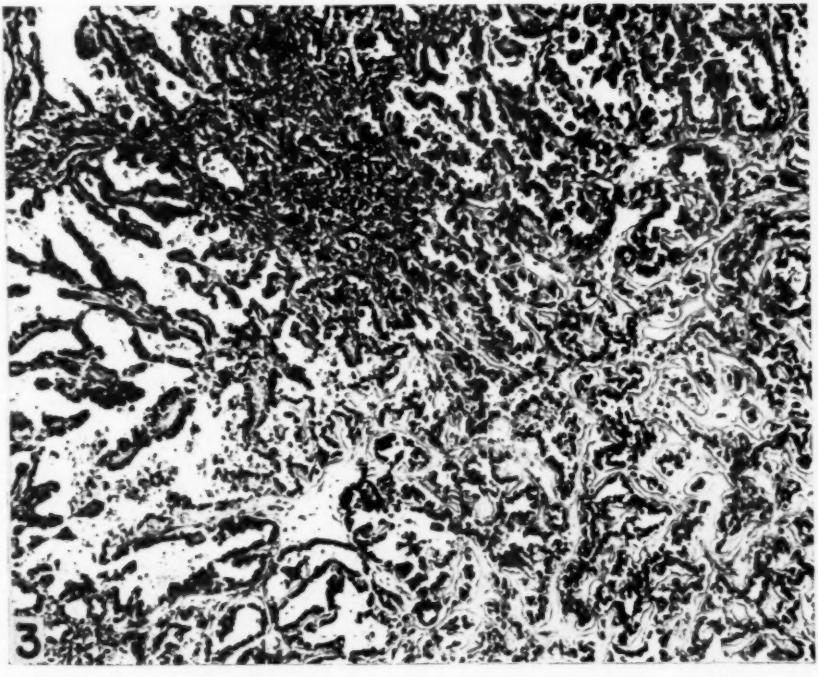

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PLATE 39

FIGURE 4.—Adenopapillomatous growths consisting of goblet cells. Hematoxylin and eosin. × 290

FIGURE 5.—Small polypoid growth in the left ovary of the tumor-bearing rat. Neoplastic adenomatous, tubular, and papillary formations are attached to the ovary by a narrow pedicle. Wilder's silver impregnation method for reticulum. × 96

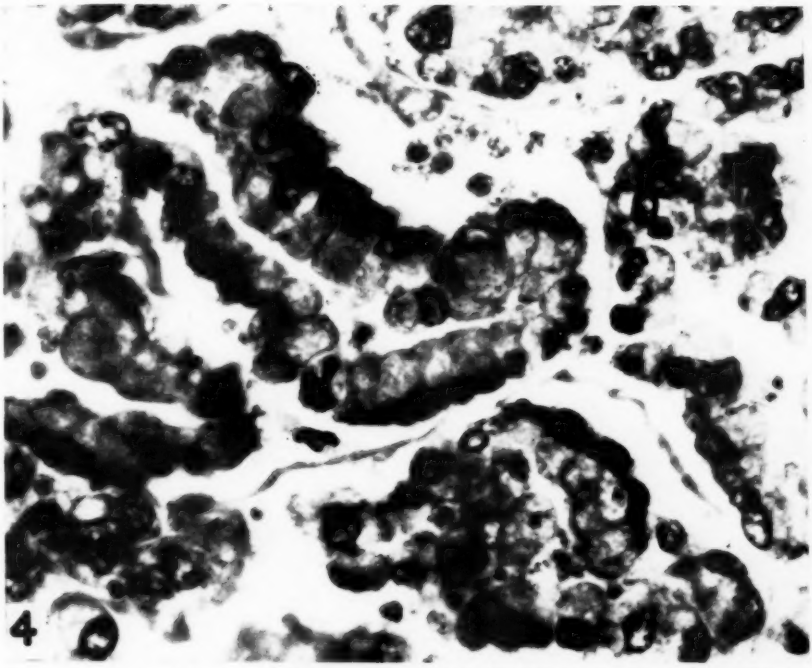

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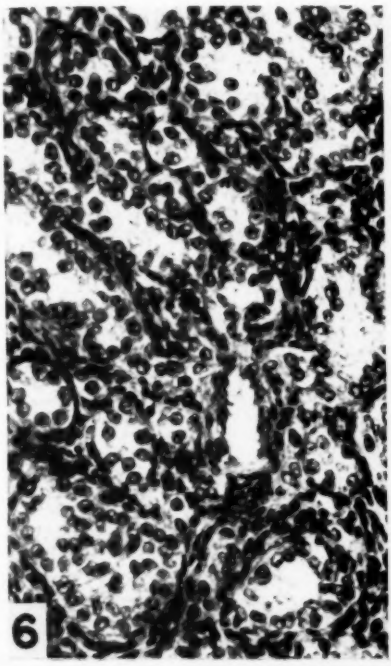
PLATE 40

FIGURE 6.—Area of polypoid growth in the left ovary with tubular and adenomatous structures, some of which resemble rudimentary ovarian follicles, surrounded by cellular ovarian stroma. Hematoxylin and eosin. × 290

FIGURE 7.—Area from the same section as shown in figure 6. Cordlike and tubular structures can be seen. Dilated formations show papillary invaginations. Hematoxylin and eosin. × 215

FIGURE 8.—Tubular structure of the polypoid growth in the left ovary resembling an aspermatic male tubule. Hematoxylin and eosin. × 315

FIGURE 9.—Large spherical tumor of the left ovary and a smaller tumor of the right ovary removed from a rat possessing intraperitoneally transplanted ascites fluid. Below the tumors, the ovaries and uterine horns of a normal tumor-free rat of the same strain and age are shown for comparison. ¾ natural size.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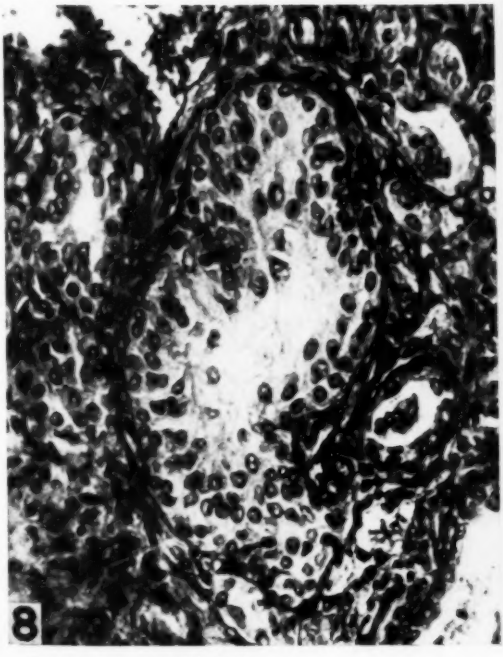
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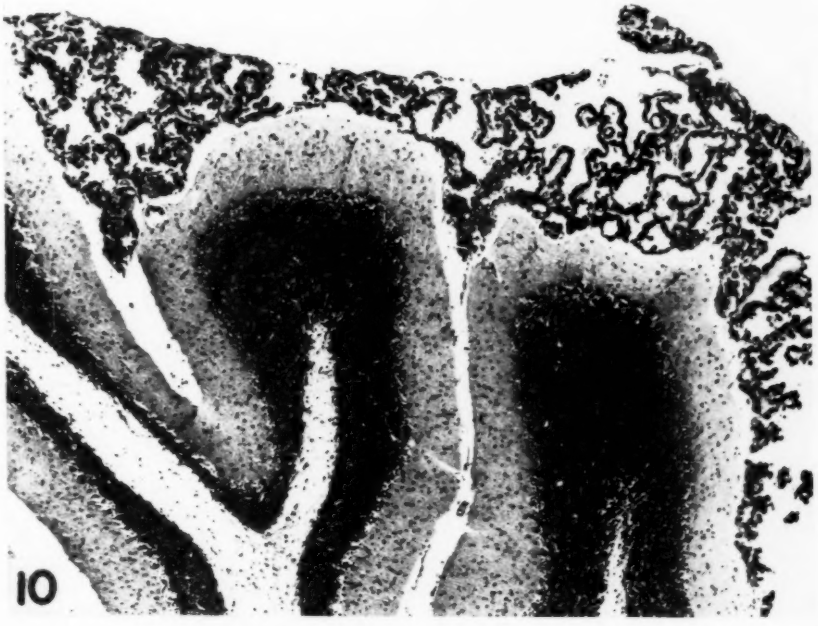
PLATE 41

FIGURE 10.—Papillary proliferation of the tumor on the meninges of the cerebellum of a Sprague-Dawley rat which had received an intracranial implant of tumor ascites fluid. Hematoxylin and eosin. × 100

FIGURE 11.—Tumor nodule on the peritoneal surface of a rat which received a transplant intraperitoneally. Thin strands of the mucoid substance, among the glandular formations, stained purplish red and appear black. Periodic acid-Schiff reaction. × 210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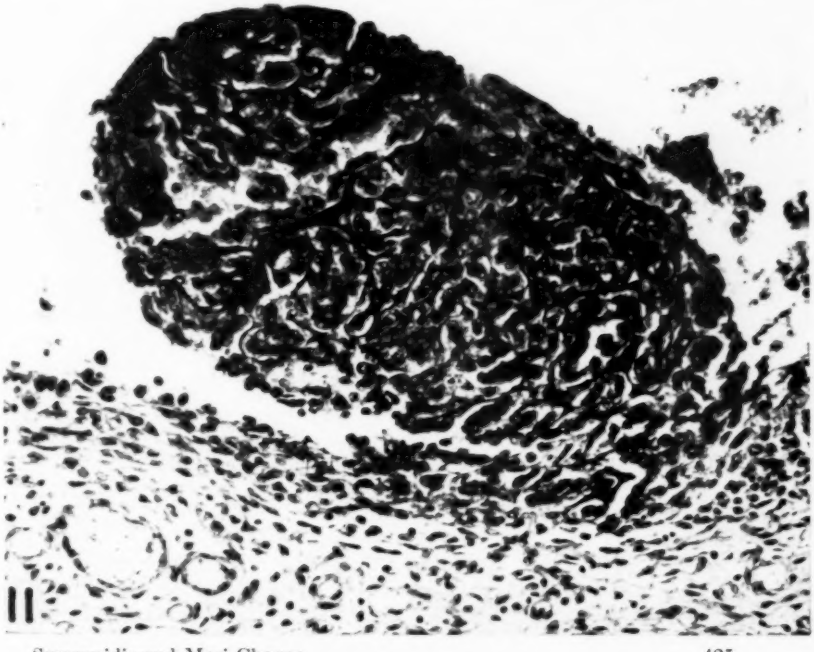
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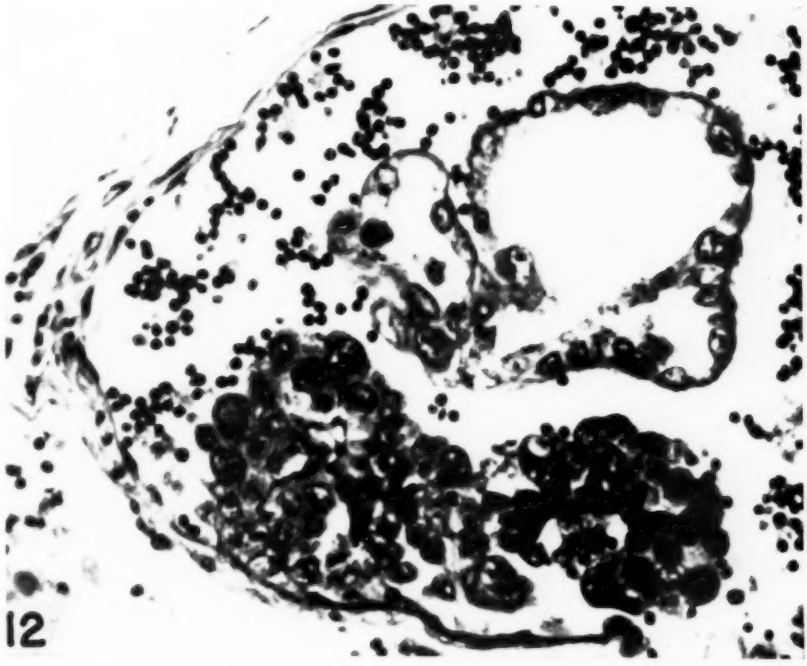
PLATE 42

FIGURE 12.—Tumor metastasis in the subcapsular sinus of a mediastinal lymph node. Note clusters of tumor cells resembling glandular structure (upper cluster). Below this, the black mucoid substance can be seen inside a number of the cells, and at the right it can be seen as distorted ropelike strands between cells. Periodic acid-Schiff reaction. × 430

FIGURE 13.—Adenopapillary tumor growths in the free space of the ovarian capsule. At the upper right, a tumor nodule with characteristic adenopapillary structure may be seen. The ovary itself was not invaded by the tumor. Hematoxylin and eosin. × 40



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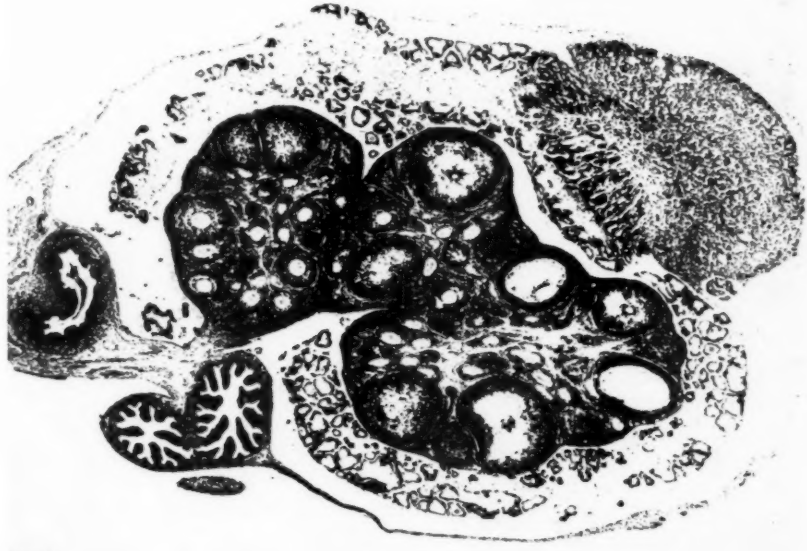

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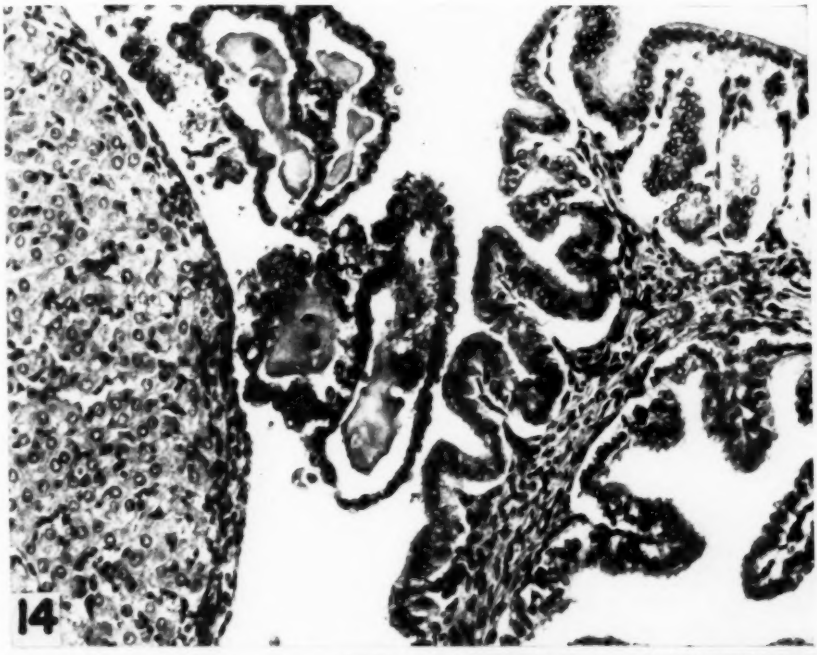
PLATE 43

FIGURE 14.—Adenopapillary growths within the ovarian capsule between a fimbria of the oviduct (right) and a corpus luteum of the ovary (left) in a rat that possessed an intraperitoneal transplant. Hematoxylin and eosin. × 235

FIGURE 15.—Ovary invaded by the tumor tissue. Evidence of partial replacement of luteal cells may be seen in center. Hematoxylin and eosin. × 75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A Quantitative Study of the Effects of X Radiation on Cells *in Vitro* [1, 2]

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The use of tissue culture as a mechanism for the study of the effects of ionizing radiations on biological systems has been attempted by a number of investigators, (*e.g. 1, 2*). In most cases, the problem has been approached by using slide cultures of the hanging-drop variety, which are advantageous for the study of individual cells and for observing the changes in mitosis induced by irradiation. They require, however, frequent subcultivation, thus making quantitative studies of cell populations impossible. Recently, Evans, Earle and Sanford (*3*) have developed a technique for the preparation of replicate tissue cultures and the quantitative determination of the number of viable cells in each culture (*4*). The application of these techniques to the study of the effects of X radiation in terms of surviving cell populations affords a system whereby a more accurate measurement of lethal effects may be made than by counting apparently degenerate cells in slide cultures. In addition to this, the cultures may be maintained over prolonged periods of time without subcultivation.

MATERIALS AND METHODS

The tissue used in all experiments was strain L mouse fibroblast obtained from the laboratory of Wilton R. Earle at the National Cancer Institute. This strain originated from a single cell isolated in 1948 (*3*) and represents a genetically homogeneous cell type. It has been made available to laboratories equipped to handle tissue cultures.

For a detailed discussion of the technique of implantation of replicate tissue cultures and enumeration of cell nuclei, reference is made to the publication of Evans *et al.* (*3*) and Sanford *et al.* (*4*). These techniques were used by the authors without any basic modification. Briefly, the

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[2] Supported in part by funds made available by the Atomic Energy Commission.
[3] *Present address:* Department of Radiology, U. S. Public Health Service Hospital, Baltimore, Maryland.
[4] National Institutes of Health, Public Health Service, Federal Security Agency.

method was as follows: a suspension of strain L fibroblasts in horse serum was dispensed in 0.6 cc. or 0.4 cc. amounts, while under continuous agitation by stirring, into tissue culture flasks having a floor area of 6.5 cm.^{2}, but otherwise in design identical to the T-12 flask (*3*) used by Sanford and Evans. The volume of the suspending fluid varied according to the number of flasks to be inoculated from the parent cultures, but the final volume in each flask was adjusted, by the addition of the necessary components of the medium, to 1 cc. The growth medium consisted of a 2:2:1 mixture of filtered horse serum, balanced saline solution, and 1:1 mixture of chick embryo juice saline prepared from 9-day chick embryos. Following inoculation, the cultures were incubated at 37.5° C. and 48 hours later, one-half cc. of the 2:2:1 medium was added to each culture. Thereafter, the fluid medium was changed three times a week in the following manner: the flasks were first centrifuged at 500 r.p.m. for 20 minutes, then transferred in an upright position with a minimum of agitation to a rack which holds 8 flasks. In a sterile room, the necks of the flasks were flamed, used 00 rubber stoppers removed, and 1 cc. of old medium aspirated by means of a 1 cc. volumetric pipette connected to a greased 2 cc. syringe. One cc. of fresh 2:2:1 medium was added, and the flasks were agitated gently to break up the clump of free cells in the bottom, restoppered, and placed back in the incubator in a horizontal position.

The initial inoculum of cells was usually 0.2 to 0.4 million per flask and at 3 to 5 days following inoculation the individual flasks contained from 0.6 to 1.0 million cells. At this point, the cultures were irradiated in a circular incubator chamber constructed to hold 8 cultures at one time arranged radially in 2 nonoverlapping layers. The radiation incubator (fig. 1) consists of an outer water jacket constructed of 1/16″ rolled copper, which contains a flexible "Lo-Lag" copper-sheathed 500 watt immersion heating unit [5] wound about the entire periphery of the jacket. The heating element is operated at 0.9 amps. by means of an adjustable transformer. A mercury thermoregulator [5] adjusted to 37° C., mounted inside the air chamber central to the water jacket, controls the temperature of the air inside the chamber to 37°±0.2° C. by means of a relay system. Two 1/8″ thick lucite disks enclose the air space inside the water jacket and the cultures are supported by means of lucite racks attached to the bottom disk. The upper disk is removable for loading of the cultures.

The radiation conditions were as follows: the incubator with the cultures inside was placed midway between two tubes (fig. 2) mounted in juxtaposition 50.5 centimeters apart. The tubes are operated at 186 KVP, 20 milliamperes; additional filtration, 0.55 mm. aluminum plus 0.25 mm. copper; dose rate 380 r per minute ±5%. Measurements made with a Victoreen chamber, in a phantom constructed to simulate conditions of scatter and filtration found inside a culture flask in the incubator chamber under the above radiation conditions, showed a phantom/air ratio of 1.03. All subsequent measurements, which were made routinely prior to irradiation, were made in air and this correction factor applied.

[5] American Instrument Co., Silver Spring, Maryland.

The method of preparation of the cell nuclei for counting was as follows: The cultures were fixed in 0.1 molar citric acid at 37° C. for 1 hour during which time they were shaken several times. The nuclei were then stained with 0.1% crystal violet, transferred to a 10 cc. graduate and, following centrifugation, the volume of fluid quantitatively adjusted. An equal volume of 0.5% methylcellulose (4,000 c.p.s.) in 0.1 molar citric acid was added and an aliquot of the resuspended mixture counted in a hemocytometer having a fluid volume of 0.9 cubic millimeter. Two samples from each culture were counted by each of the authors and the results combined to determine the number of nuclei per culture according to the formula:

$$\frac{\text{Nuclei counted} \times \text{volume of suspension (cc.)}}{\text{Volume of hemocytometer (3.6 cu. mm.)} \times 1{,}000}.$$

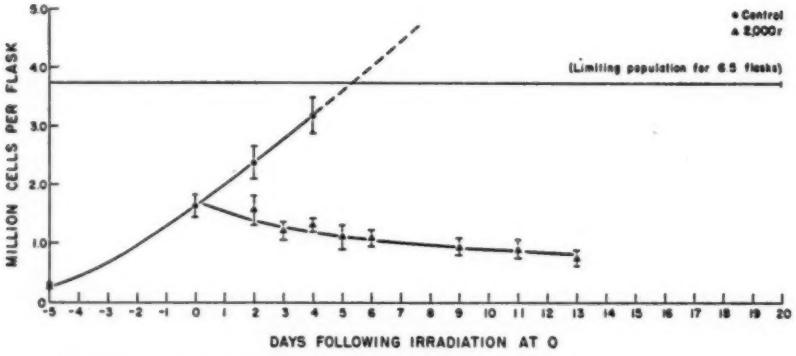
Sanford (*4*) states that by this method only nuclei of viable cells and those in early stages of degeneration are preserved intact for counting. It is possible to recognize cells in early stages of degeneration by the lack of staining of their nuclei. In this study, only the nuclei of healthy cells were counted.

EXPERIMENTAL RESULTS

The purpose of this study was to determine the dose-response pattern with various dosages of X radiation, in order that a reference point might be established for further work in radiation physiology using this method. Therefore, the only variants were the dose of radiation and the unavoidable variation in the size of the initial inoculum from one experiment to another. There was, of course, qualitative variation in the composition of the horse serum and embryo extract since different lots of each were used. This factor is reflected in the different rates of growth of the cultures in the various experiments; however, the size of the initial inoculum also has an important bearing on this phenomenon. Qualitative variations in the horse serum and embryo extract might well have been overcome by pooling a large supply of each, but the resultant mixtures would not be reproducible. It is felt that the patterns of response to radiation obtained are sufficiently reproducible that the results may be applied to future work without too much concern with minor variations in media.

The dosages of radiation given were 250 r, 500 r, 750 r, 1,000 r, and 2,000 r, and each experiment was repeated several times.

2,000 r.—The pattern of response to 2,000 r is shown by the curve in text-figure 1. It will be noted that there is an arrest of growth for the first 24 hours postirradiation followed by a slow, continuous falling off of the cell population to the point where at 13 days the population is about one-half of what it was at the time of irradiation. This pattern was confirmed by three other experiments, one of which was carried out for a period of 22 days following irradiation. In none of these experiments could recovery be demonstrated; therefore, this apparently represents a totally lethal dose. At 48 hours, and thereafter, the nuclei of the irradiated cultures showed rather marked swelling and poikilocytosis in comparison



TEXT-FIGURE 1.—Dose-response following 2,000 r.

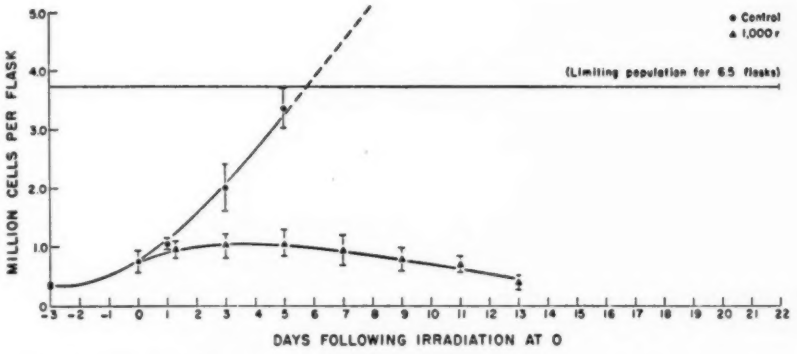
with the controls. There was no evidence of mitosis throughout the entire observation period. However, no quantitative determinations were made to compare the mitotic activity in the controls to that in the irradiated cultures.

The maximum cell population which can be obtained in a 6.5 flask is 3.5 to 3.75 million cells. After this limiting population is reached, there is an abrupt decline in the number of viable cells due to necrosis attendent upon overcrowding. For this reason, the control curves are extrapolated for the portion beyond the 3.75 million mark (broken line) to represent the growth pattern if there were adequate space available for growth. Each point on the 2,000 r curve represents the average of the cell populations of 8 flasks. The average constant variation of all the control cultures in text-figure 1 is $\pm 6.5\%$, while that of the experimental cultures is $\pm 5.3\%$.

1,000 r.—Text-figure 2 shows the dose-response curve for 1,000 r. Unlike the 2,000 r series, the irradiated cultures continue to proliferate for 4 to 5 days following irradiation, but at a much diminished rate compared to the controls. After 5 days, the population begins to fall off slowly until at 13 days it is about equal to that at the time of innoculation (3 days before irradiation). In another experiment in which the irradiated cultures were maintained for 22 days following irradiation, this pattern is repeated, and no recovery was demonstrable. The net effect, therefore, is identical with that seen with 2,000 r.

The average constant variation in the 1,000 r experiment illustrated was $\pm 6.8\%$ for the controls and $\pm 10.9\%$ for the irradiated.

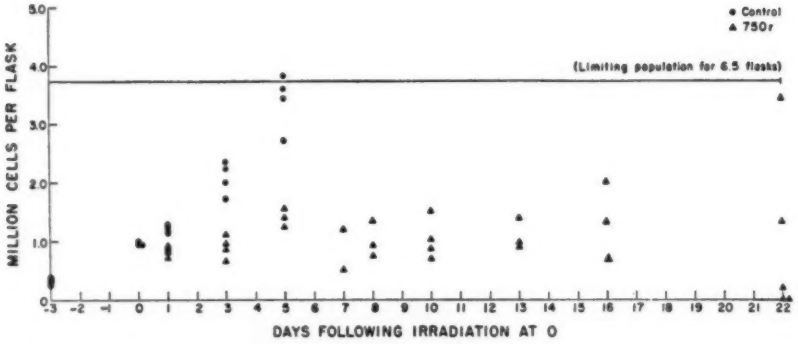
750 r.—In the above experiments the pattern of response has been quite clearcut, *i.e.*, there has been a steady decline in the cell population. Following irradiation with 750 r (text-fig. 3), however, the response varies considerably from flask to flask. It is impossible to draw a representative curve for the period after 10 days postirradiation, due to the fact that some flasks went on to apparent complete recovery whereas others showed a decline in cell population to zero. For the first 10 days following


• Control
▲ 1,000 r
(Limiting population for 6.5 flasks)
MILLION CELLS PER FLASK
5.0
4.0
3.0
2.0
1.0
0
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
DAYS FOLLOWING IRRADIATION AT 0

TEXT-FIGURE 2.—Dose-response following 1,000 r.

irradiation, the values for 4 flasks counted at any one time were quite closely correlated, *e.g.*, on the third day following irradiation the variation in the counts was from 0.68 million to 1.1 million and on the fifth day was 1.26 to 1.56 million, so that the mean value at these points is representative. If this were true, the mean population shows a rise following irradiation which is greater than that observed at 1,000 r. However, at 16 and 22 days the spread of values becomes so great, due to beginning death of the cultures, that the mean value is no longer truly representative of what is taking place. For this reason, the values for each flask are plotted in text-figure 3. At the time the experiment was terminated (22 days), one flask had proliferated to a point (3.48 million) approximating the limiting population, 2 flasks showed no viable cells, and 2 others lay between with values of 0.229 and 1.34 million. It is probably safe to assume that if the flask having a population of 1.34 million had been allowed to grow, it too would have shown complete recovery. In the light of the above results, it may be concluded that 750 r represents a threshold dose from which approximately one-third to one-half of the cultures will recover. The average constant variation for the control series in this experiment was $\pm 4.54\%$.

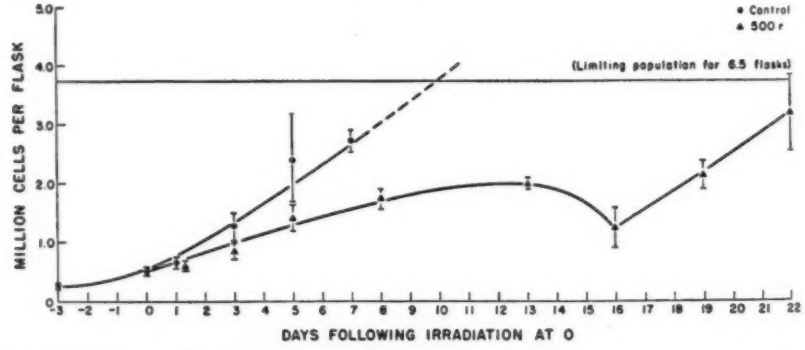
500 r.—As might be expected from the results at the dose level of 750 r, when one administers 500 r there is a complete recovery, which is manifested by a return of the population curve of the irradiated series to a slope approximating that of the controls. As seen in text-figure 4, the sequence of events following irradiation is an initial slow rise in population followed by a decline or plateau, then rapid proliferation. This pattern has been reproduced in 3 separate experiments, and varies only in that the time following irradiation at which recovery occurs is not constant. If one arbitrarily takes the low point of the recovery portion of the curve which follows the initial rise as the time when "normal" proliferation begins, this point was found to be at 7 days postirradiation in one experiment, 10 days in another, and 16 days in the one shown in text-figure 4. The slopes of the control curves in these experiments were found to be



TEXT-FIGURE 3.—Dose-response following 750 r.

progressively less steep, *i.e.*, the control slope in the experiment in which the low point occurred at 7 days was greatest and where the low point occurred at 16 days it was least. Hence, it might be inferred that the time of recovery is a function of the metabolism of the cultures. The size of the initial inoculum was the same in the latter two above and only 30,000 more in the first, so the different rates of growth are due, in all probability, to variations in the chemical composition of the media. The average constant variation in the controls for the above 3 experiments was ±7.3%, ±8.0%, ±4.5%, while in the irradiated series it was ±6.4%, ±7.0%, ±10.2%.

250 r.—In five experiments performed at this dose level, it was found that the response varied from a very slight lag in growth, lasting about 24 hours, to one of no significant effect whatsoever. The results were not reproducible so one is forced to conclude that with these techniques there is no demonstrable effect with 250 r. There is undoubtedly some effect which might be reflected by a transient depression in the mitosis as shown by Lasnitzki (*6*), but it is apparently not so profound as to manifest itself by a change in the total population.



TEXT-FIGURE 4.—Dose-response following 500 r.

DISCUSSION

The results described above provide a rather well-defined baseline from which further work might be projected along lines of modification of radiation effects or studies of their mechanisms.

The response to 500 r is of special interest, since the shape of the irradiation curve indicates that following an initial brief lag of about 24 hours, there is continued growth followed by death of a large portion of the cell population, as indicated by the plateau or decline in the curve. If there were simply a lag in mitosis followed by recovery of most of the cells, the irradiated curve would show an initial plateau immediately following irradiation. After such a lag period, proliferation would proceed at a rate comparable to the controls. Since this is not the case, one must postulate that a great number of the cells are so affected by 500 r as to cause death after several divisions. Strangeways and Hopwood (7) showed that cells radiated just prior to prophase with low doses of gamma radiation will undergo abnormal mitosis followed by disrupture of the cells. However, since only approximately 0.5% of the total population of the strain L fibroblasts are in mitosis at any one time, it is difficult to visualize how, if only the cells in any one stage of the cycle were affected, there would be enough lethal effect to cause the amount of cell death which occurs. Therefore, one might postulate that there is some type of lethal mutation which occurs in a large proportion of the population following 500 r, while the remainder escape and eventually repopulate the culture to the extent that complete recovery is demonstrable on a numerical basis. If one extrapolates the recovery portion of the irradiated curve in text-figure 4 back to the thirteenth day on the rough basis of a doubling of the population in 3 days, one notes that the intercept is at about 0.6 million. Therefore, the difference between this point and the irradiated population value (2.0 million) represents the number of cells which are destined to die. In this case the number is 1.4 million or 75% of the population. The 0.6 million cells, which have been temporarily inhibited, begin to multiply after 13 days and repopulate the culture flasks. At the higher dose levels of 1,000 and 2,000 r, the effect is so pronounced that no cultures were able to recover, although some cells in most cultures remained viable for as long as 3 weeks, thus showing that there is a cell-to-cell variation even with doses of a magnitude that is lethal by our criteria.

SUMMARY

Results of the application of a quantitative method for the determination of cell population in tissue cultures to studies of dose-response patterns following X irradiation is reported. The response to 250, 500, 750, 1,000, and 2,000 r is described. It was found that recovery occurs following doses of 250 and 500 r; whereas 1,000 and 2,000 r are lethal, and 750 r represents a partially lethal dose.

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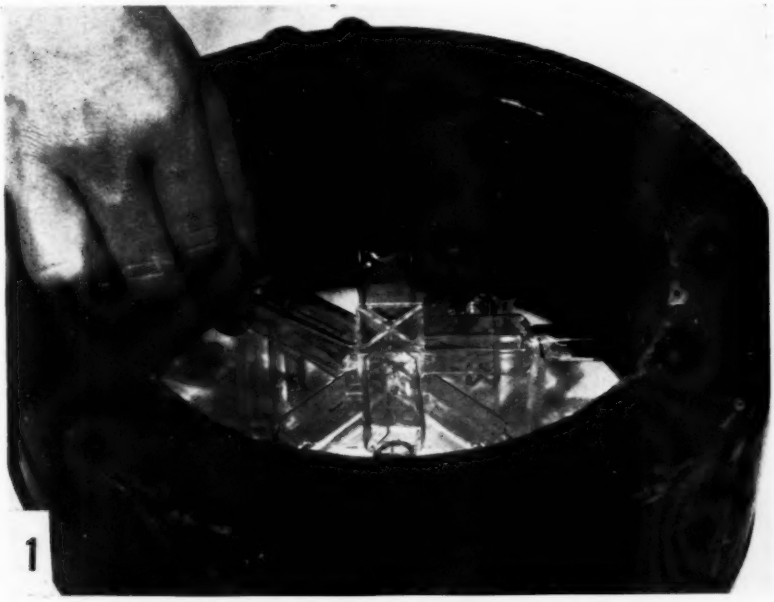
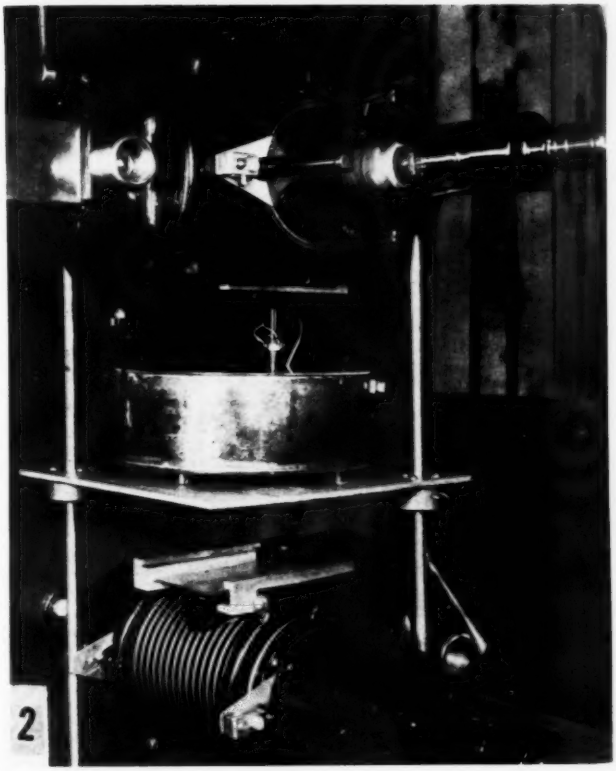
PLATE 44

FIGURE 1.—Radiation incubator with tissue culture flasks in place.

FIGURE 2.—Incubation chamber in position for irradiation.





216903—52——22

Squamous-Cell Carcinoma of the Forestomach in X-Irradiated Mice Fed 9,10-Dimethyl-1,2-Benzanthracene, with a Note on Failure to Induce Adenocarcinoma [1]

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Carcinoma of the glandular stomach has not been induced by feeding carcinogenic hydrocarbons (*1*). The possible effect of a mucous barrier has often been suggested (*2*), and various solvents have been used without success in attempts to change the refractoriness of this tissue to the induction of carcinoma (*3*, *4*, *5*). The failure of bile when combined with methylcholanthrene to induce gastric tumors in mice has also been reported (*6*). Penetration of a carcinogenic hydrocarbon into the wall of the glandular stomach has been observed by the author when association colloids were used as solvents (*5*). This observation was later confirmed (*7*).

In the present experiment an attempt was made to alter the function and structure of the glandular stomach by X irradiation of the gastric region. It was believed that a possible alteration of the glandular mucosa might result in a lesion which would provide a focus for penetration of a carcinogen subsequently fed to a mouse. Although no tumors were produced in the glandular stomach during the time the mice survived, certain other changes developed that are of interest.

MATERIAL AND METHODS

One hundred and ninety 3-month-old strain C57BR mice of both sexes were used. X irradiation was given on the ventral side over an area 1 × 2 cm. in width in the upper abdominal region. The irradiation resulted in a depigmented area of hair encircling the body. Physical factors of irradiation were: 186 KVP, 20 ma., additional filter: 0.25 mm. Cu, and 0.55 mm. Al, focus-skin distance 35 cm., output 104.8 r.p.m.[4]

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The carcinogenic hydrocarbon employed was 9,10-dimethyl-1,2-benzanthracene in an aqueous solution of the association colloid Triton X-100 (5% solution with 300 mg. 9,10-dimethyl-1,2-benzanthracene per 1,000 ml.). A dose of 0.3 ml., equal to 0.09 mg. carcinogen, was administered 3 times weekly for 10 weeks. The carcinogen was introduced directly into the stomach by means of a thin polyethylene cannula. The animals were given Purina laboratory chow and water *ad libitum*.

The mice were divided into seven groups as follows: 1) carcinogen given without previous irradiation, 2) carcinogen given after 500 r, 3) carcinogen given after 600 r, 4) carcinogen given after 1,000 r, 5) irradiation with 600 r and no carcinogen, 6) irradiation with 1,000 r and no carcinogen, and 7) Triton solution given without carcinogen after irradiation of 600 r.

In the groups receiving irradiation alone, the mice were killed at different intervals following irradiation. In the other groups, the animals that appeared to be sick were killed and all remaining mice were killed at 6 months following initiation of the treatment. The animals were killed with ether and the tissues fixed in Zenker-formol fluid. After fixation, the stomach was incised along the greater and lesser curvatures, and entire cross-sections were taken for histologic study. The tissues were embedded in paraffin and the sections stained with hematoxylin and eosin, and prepared by the periodic acid-Schiff method.

RESULTS

Changes produced by X irradiation in the glandular stomach.—The glandular mucosa of irradiated mice killed at different intervals after irradiation was compared with the normal mucosa (fig. 1). After irradiation with 600 r, minor changes in the chief cells were observed. After 1,000 r, the changes were definite, and 1 week later, slight edema, swelling and vacuolation of the cytoplasm of the chief cells occurred (fig. 2). A few inflammatory cells were found below the mucosa at this interval following irradiation, and 2 and 4 weeks after irradiation there was considerable accumulation of inflammatory cells. The chief cells were reduced in number but more than the usual number of mucous neck cells and parietal cells were apparent (figs. 3, 4). After 6 weeks there were still very few chief cells, and the other cell types appeared to be normal. Two months after the irradiation many sections showed no chief cells, and at this time the parietal cells and mucous neck cells were also greatly reduced in number. The glands seemed to be lined with surface epithelial cells (figs. 5, 6). During the third month, the mucous neck cells were increased in number, and chief cells and parietal cells were also found in greater number. The only changes found after the third month were an occasional increase of connective tissue between the glands, and squamous metaplasia (figs. 7, 8). No single case showed ulceration of the glandular stomach.

Gastric tumors produced.—As mentioned above, no tumors were produced in the glandular stomach. The incidence of tumors of the fore-

stomach is shown in table 1. In the animals that were irradiated and fed carcinogen, there were 40 female and 38 male mice that died or were killed between 4 and 6 months after the irradiation. In the 40 female mice, 21 carcinomas, 3 nearocarcinomas (*8*), and 12 papillomas were found in the forestomach. The corresponding figures in the 38 male mice were: 32 carcinomas, 3 nearocarcinomas and 1 papilloma. Thus, it seems that the incidence of carcinoma of the forestomach was higher in male mice. A possible explanation is that the induction time of fully developed carcinomas was shorter in male mice, since the incidence of papillomas was higher in female mice. The same difference was found in each of the three subgroups. In the group that was fed carcinogen without previous irradiation, there were 9 female and 9 male mice. In the females, 6 carcinomas, 1 nearocarcinoma and 2 papillomas were found, while the corresponding figures for males were: 6 carcinomas, 1 nearocarcinoma and 1 papilloma. Hence, no sex difference was noted. No gastric tumors were found in the control groups that received only irradiation and in the group where the irradiation was followed by feeding of the Triton solution without carcinogen.

TABLE 1.—*Tumors induced in mice 4 to 6 months after the start of the experiment; the mice were killed or they died at this time*

Experimental group	Sex	Number of mice	Tumors of forestomach				Other tumors
			Carcinoma	Nearocarcinoma	Papilloma	Sarcoma	
1 Carcinogen: without previous radiation	F	9	6	1	2		1 pulmonary.
	M	9	6	1	1		
2 Carcinogen after 500 r radiation	F	15	8	1	4		1 ovarian.
	M	15	13	1	0		
3 Carcinogen after 600 r radiation	F	13	5	2	6		1 ovarian.
	M	15	12	1	1	1	1 hemangioendothelioma of pancreas.
4 Carcinogen after 1,000 r radiation	F	12	8	0	2	1	4 ovarian.
	M	8	7	1	0		2 lymphocytic leukemias.
Total number of mice fed carcinogen after irradiation	F	40	21	3	12		
	M	38	32	3	1		
5 Radiation of 600 r only	F	5					
	M	5					
6 Radiation of 1,000 r only	F	5					1 ovarian.
	M	3					
7 Solution without carcinogen following 600 r radiation	F	9					
	M	6					

Histologically, all the 62 carcinomas were squamous-cell carcinomas similar to those described in detail by Stewart (*8*). Twenty-six of these tumors infiltrated adjacent organs and structures or metastasized. Pulmonary metastases were observed in three instances. The same criteria proposed by Stewart for nearocarcinoma were used in the present investigation. Seven sarcomatoid tumors were observed (fig. 9), with sheets of spindle-shaped tumor cells that in some cases had no relation to epithelial tumor cells. In most tumors, however, transitional forms from unquestionably squamous epithelial cells with intercellular bridges to spindle-shaped cells were found, and the pattern of the reticulum was generally that of carcinoma. No immature blood vessels characteristic of sarcoma were noted. On the basis of such observations, these tumors were considered to be modified carcinomas. In two tumors, however, areas composed of interlacing bundles of spindle-shaped cells with the reticulum in intimate relation to each cell were seen, and in one of these fibroglia could also be demonstrated. These two cases were considered to be sarcomas (fig. 10). One of these sarcomas was associated with a carcinoma.

Of other changes observed, the following should be mentioned. In two mice that died 54 days after treatment with 1,000 r and subsequent feeding of carcinogen, ulcers of the forestomach were observed. Fibrinoid degeneration of collagen was often observed in the stroma, particularly of the forestomach. The muscularis was very faintly stained in some areas and was infiltrated with inflammatory cells. Seven of the 59 female mice that received X irradiation over the stomach region developed ovarian tumors. Atrophy of the ovaries, typical of irradiation injury, was common. The ovarian tumors were of the granulosa-cell type and similar to the radiation-induced ovarian tumors in mice (*9*). Hemangio-endothelioma of the pancreas was found in one case, lymphocytic leukemia in two, and multiple pulmonary tumors in one.

DISCUSSION

The changes found in the glandular mucosa after irradiation were similar to those described in reports from other authors (*10*). A minor early reaction, chiefly edema, occurred within the first 2 days. The major effect, consisting of degenerative changes in the epithelial cells of the mucosa and in the stroma, with edema, and leukocytic infiltration began in about a week. The degenerative changes were most severe in 1 to 2 months and in previous experiments they sometimes resulted in ulceration. In the present experiment, with 600 r and 1,000 r, however, no ulceration was noted with X irradiation alone. The basal portion was more easily damaged than the surface epithelium of the glandular mucosa. The chief cells showed the most obvious damage, but the mucous neck cells and parietal cells were greatly reduced in number at a later time. After 2 months, the picture may vary with combinations of atrophy of some cells and proliferation of others, squamous metaplasia, and fibrosis.

Most of the experimental studies on the effect of radiation on the function of the gastric mucosa agree that temporary depression of acidity occurs. This seems to be in agreement with the histologic changes described above.

Although X irradiation produced lesions in the glandular stomach and probable changes in the function of the stomach, treatment with the carcinogen did not alter the glandular stomach of mice.

Tumors of the forestomach were generally similar to other tumors induced by carcinogens in this region; these have been well described (*8*). It may be of interest that 7 carcinomas were found with sarcomatoid areas in the irradiated animals and that two spindle-cell sarcomas of the wall of the forestomach were present. Sarcoma has rarely been induced by feeding carcinogen alone (*6*). Lorenz and Stewart (*3*, *4*) reported that of 425 mice fed oily solutions of carcinogen, none developed sarcomas of the forestomach. Neither did sarcomas develop in earlier experiments in which Triton X-100 was used as the solvent (*5*). It is possible, therefore, that X irradiation did have a particular effect in the development of the sarcomas of the forestomach in the mice of the present experiment.

Sarcomas developing in the skin after painting are presumed to be due to the penetration of the carcinogen into the subepidermal tissues. Sarcoma of the stomach in mice can be easily induced by intramural injection of the carcinogen (*1*). The question may be asked whether irradiation alters the mucosa in such a way that the carcinogen penetrates it more easily, or whether the underlying stroma is so altered that the carcinogen remains longer in contact with susceptible cells. It should be noted that the sarcomas in mice of the present experiment were in the wall of the forestomach and that ulcers of the forestomach were observed in two mice that died 54 days after the start of feeding carcinogen. Changes involving the muscularis and the connective tissue stroma, especially that of the forestomach, were also often found.

It is noteworthy that the incidence of carcinoma of the forestomach following X irradiation was considerably lower in females than in males. Only 21 of 40 females developed carcinoma, whereas 32 of 38 males showed carcinomatous growth. On the other hand, 12 papillomas were found in the females and only one in the males. It may be that the time required for the fully developed squamous-cell carcinoma is longer in females so that the papillomas persist for a longer period. In the control group without irradiation, no sex difference in tumor incidence was observed, and no mention of a sex difference has been reported in the literature in carcinomas induced in the forestomach. In the present experiment, all of the animals were killed or died between 4 and 6 months after the beginning of the experiment. In earlier experiments reported in the literature, the time varied from 194 to 273 days. Therefore, the possible sex difference, if due to a shorter induction period in male mice, could have been missed because all of the tumors had developed into carcinomas.

In considering the sex difference, it must also be borne in mind that when the upper part of the abdomen is irradiated not only the stomach but also

the liver, spleen, pancreas, adrenal glands, coeliac ganglion and ovaries may receive irradiation while the testes are nonirradiated. What the secondary effect of this is on the stomach is unknown. The production of ovarian tumors is of interest and indicates that the ovaries received radiation. It has long been known that ovarian tumors develop in mice exposed to X irradiation. It has been assumed that both an indirect hormonal mechanism and a direct local effect of the irradiation on the ovaries are responsible for the formation of tumors in the ovary. It has also been reported that local irradiation of a single ovary yields a neoplasm only if the opposite ovary is excised or is also irradiated. More recently, Kaplan (*9*) concluded that secretion of ovarian endocrine glands inhibited the development of tumors in irradiated ovarian grafts. The possible sex difference in the tumors of the stomach in the present experiment might thus be due to a disturbance in endocrine relationships in females caused by radiation of the ovaries.

No tumors of the intestine were produced in this experiment nor in the earlier experiments (*5*) where aqueous solutions of Triton have been used as solvents. In experiments with oil emulsions, they have been fairly common (*4*). This is consistent with observations made on the fluorescence of carcinogens in the gastric mucosa (*5*), which reveal a more rapid disappearance when Triton solutions were used than when oil emulsions were used.

Since spontaneous adenocarcinoma of the stomach in mice is almost nonexistent, and since orally administered carcinogens have so far failed to produce gastric adenocarcinoma, it would be well to give consideration to some possibly essential anatomic differences between the stomach of mice and that of man. Heterotopic islands of intestinal epithelium have been suggested as the foci in which the majority of carcinomas in human beings originate (*11*). Since such foci have not been reported in mice, this may be a possible reason for the difference.

SUMMARY

X irradiation with 500, 600, and 1,000 r of the stomach region of 110 C57BR mice and subsequent feeding of 9,10-dimethyl-1,2-benzanthracene in aqueous solution of Triton X-100 failed to induce tumors in the glandular stomach in six months' time. Squamous-cell carcinoma of the forestomach developed in a high percentage of the mice. The incidence of carcinoma of the forestomach following irradiation was lower in females than in males.

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PLATE 45

Changes produced in the glandular mucosa of the stomach of mice by X irradiation (1,000 r). The sections are from the greater curvature near the limiting ridge.

FIGURE 1.—Normal glandular mucosa. Hematoxylin and eosin. × 300

FIGURE 2.—Glandular mucosa 1 week after irradiation. The chief cells are swollen and reduced in number. Hematoxylin and eosin. × 300

FIGURE 3.—Glandular mucosa 4 weeks after irradiation. No chief cells are seen, but mucous neck cells and parietal cells appear to be more numerous. Leukocytic infiltration at base of glands. Hematoxylin and eosin. × 180

FIGURE 4.—Glandular mucosa 4 weeks after irradiation. Mucous neck cells are clearly visible. Periodic acid-Schiff reaction. × 220

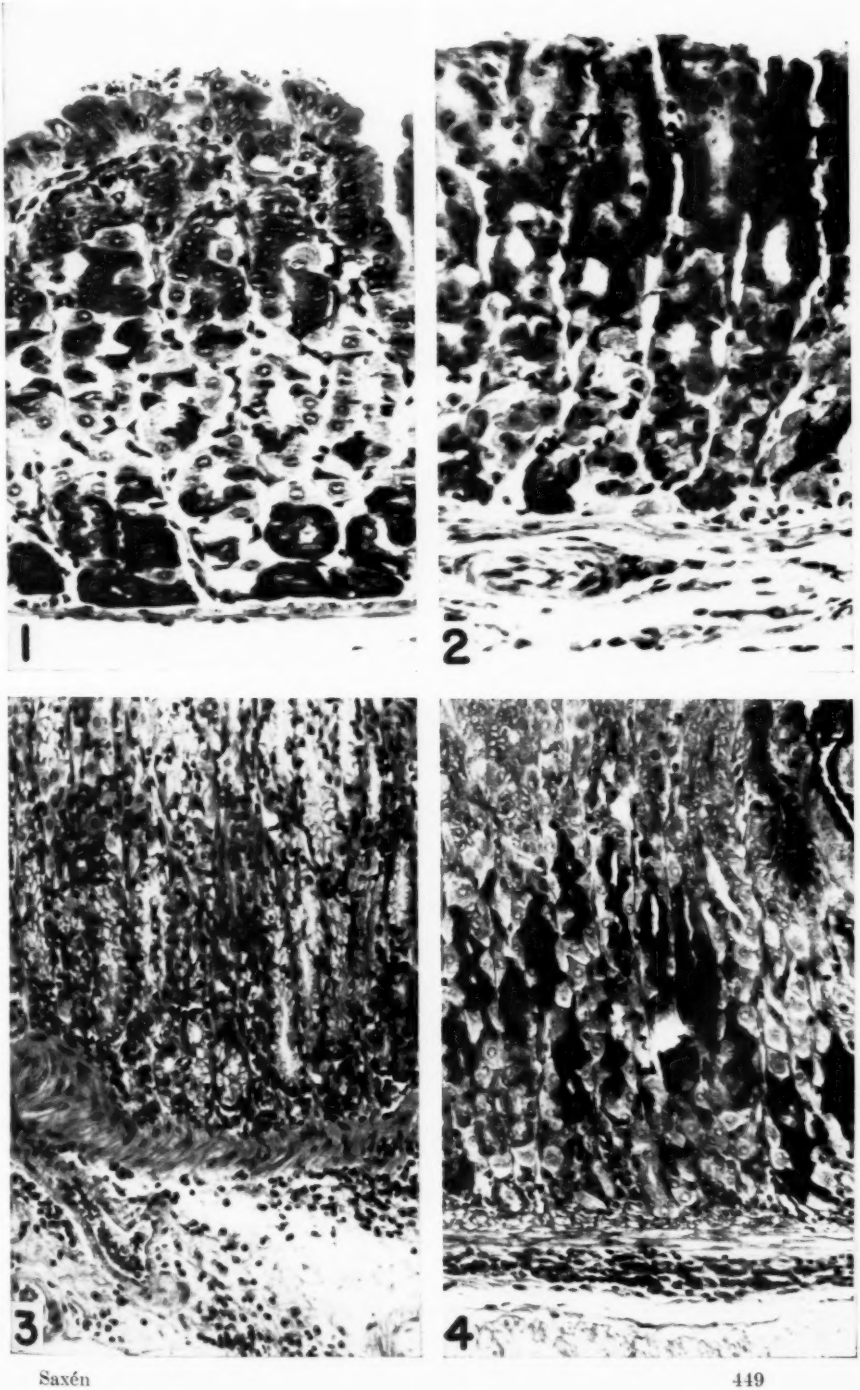

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PLATE 46

Changes produced in the glandular mucosa by X irradiation (1,000 r). The sections of figures 5 and 6 are from the greater curvature near the limiting ridge.

FIGURE 5.—Glandular mucosa 8 weeks after irradiation. The different cell types cannot be distinguished. Hematoxylin and eosin. × 250

FIGURE 6.—Glandular mucosa 8 weeks after irradiation. Very little mucoid material and no mucous neck cells are shown by periodic acid-Schiff reaction. × 300

FIGURE 7.—Glandular mucosa 12 weeks after irradiation. Squamous metaplasia near the pylorus. Hematoxylin and eosin. × 500

FIGURE 8.—Glandular mucosa 12 weeks after irradiation. Increase in connective tissue. Hematoxylin and eosin. × 260

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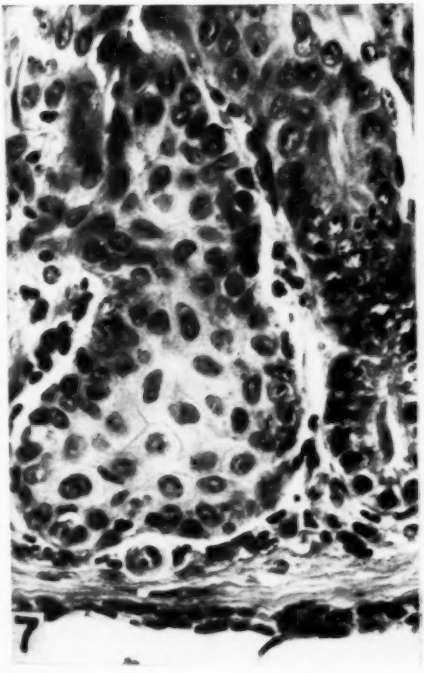
6



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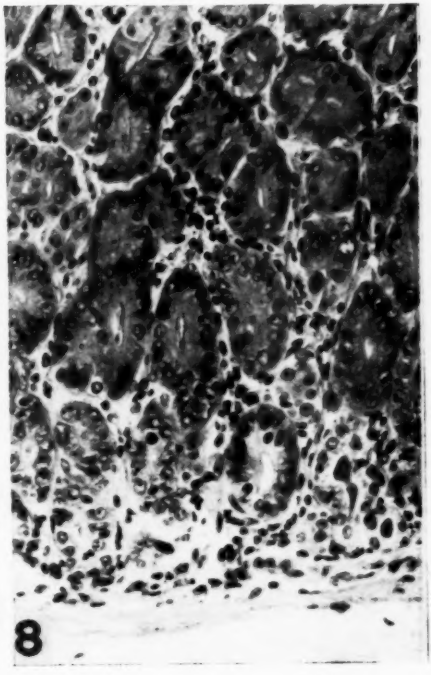

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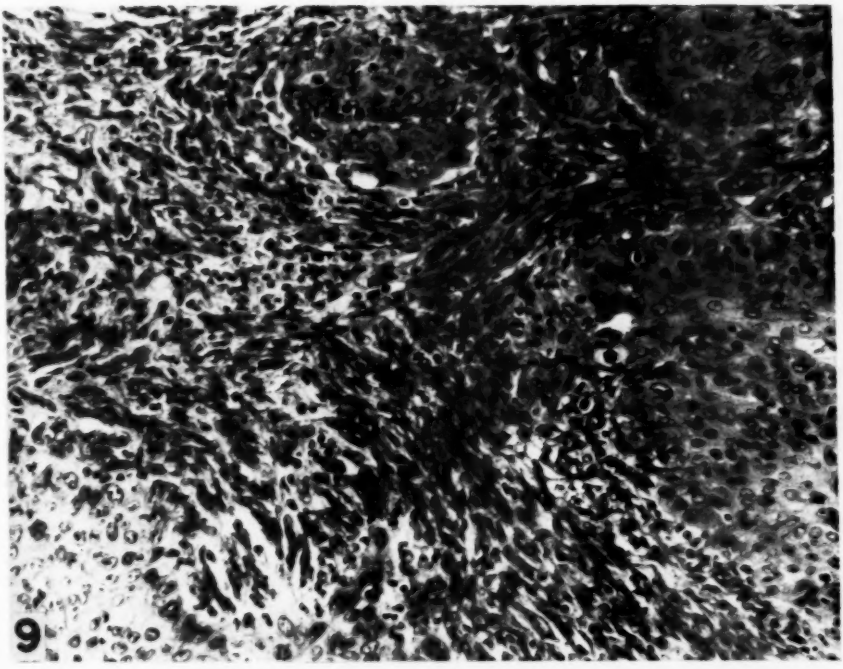
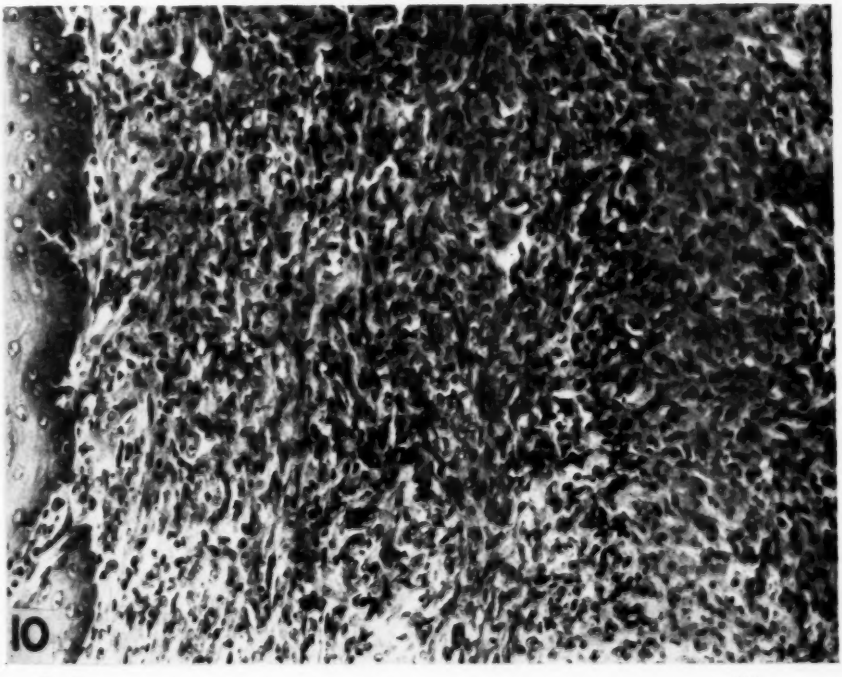
PLATE 47

FIGURE 9.—Sarcomatoid carcinoma of the forestomach. Note islands of round carcinoma cells among the fusiform cells. Hematoxylin and eosin. × 200

FIGURE 10.—Sarcoma of the wall of the forestomach. Hematoxylin and eosin. × 260





216903—52——24

Transplantation of Spontaneous and Induced Hepatomas in Inbred Mice[1]

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Most attempts to transplant primary hepatomas of mice into genetically susceptible hosts have been unsuccessful. Strong and Smith (*1*) implanted a spontaneous hepatoma from a strain CBA mouse into the subcutaneous tissues of 16 members of the same strain but recorded growth of the tumor in only 4 of the recipients. In a subsequent paper (*2*), they reported that the tumor had undergone 15 transplant generations and had been inoculated into 131 CBA mice: the number of mice in which the tumor grew was not given. Gorer (*3*) referred briefly to a spontaneous hepatoma from a CBA mouse that had been carried through 2 transfers. Burns and Schenken (*4*) used 3 spontaneous hepatomas from strain C3H to inoculate 28 mice subcutaneously, intraperitoneally, or into the anterior chamber of the eye. None of the recipient animals had developed a tumor within 1 to 5 months after inoculation. Edwards, Dalton and Andervont (*5*) grafted a spontaneous hepatoma from a C3H animal into the subcutaneous tissues of 4 mice of the same strain; 1 had a tumor 1.0 cm. in diameter within 8 months, 1 a tumor of similar size within 13 months, and 2 were without tumor 15 months after inoculation. Each of the tumor lines was carried through serial passage: one for 5 and the other for 3 generations. The number of successful "takes" in later generations was not given.

The majority of induced hepatomas of mice have also failed to grow when inoculated subcutaneously into members of their strains of origin. Shear (*6*), Andervont (*7*), Emmart (*8*), and Morozenskaya (*9*) have attempted to transplant hepatomas induced with *o*-aminoazotoluene. Shear used 5 tumors and found that 2 were propagable. One grew in all of 3 recipients and was carried through 10 passages; the other which grew slowly in 2 of 3 hosts was transferred through 2 passages and was then discontinued. Andervont transplanted 28 tumors of which 7 had become established in new hosts within 7 months after inoculation. The final results of this study, along with observations on other hepatomas in the

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[2] National Institutes of Health, Public Health Service, Federal Security Agency.

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mouse, are presented in this paper. Emmart transplanted 4 tumors into mice of strain C3H but only 1 was propagable. This tumor grew in but 1 of 5 mice of the first transplant generation but was successfully propagated through 40 passages. During the first 6 passages, 86 mice were inoculated of which 53 grew the tumor. Morozenskaya reported that only 1 of 7 tumors grew in new hosts and this tumor developed in but 1 of 8 first-passage animals. It then underwent 37 serial passages in 270 animals of which 51 percent grew the tumor progressively.

Edwards and Dalton (*10*) induced hepatomas in mice with carbon tetrachloride, and transplanted 8 tumors into appropriate hosts. Seven tumors failed to grow from 4 to 15 months after inoculation and one grew in 2 of 4 first-generation hosts. It was then carried through 4 transfers; the number of mice used for later passage was not given.

When the data in these reports are summarized it is found that 58 primary spontaneous or induced hepatomas were transplanted into their strains of origin and 15, or 26 percent, grew in the first transplant generation. A total of 43 mice received tissues from 7 of the 15 successfully transplanted growths, and 15, or 35 percent, developed takes. It is noteworthy that 14 of the first-transplant-generation tumors were propagated successfully through at least one more transfer.

Such findings with inbred mice present an interesting problem in tumor-host relationship, for all individuals of an inbred strain should be susceptible to the growth of a tumor arising in one of their members. One explanation for the discrepancy between the 43 mice inoculated with propagable primary hepatomas and the 15 showing takes could be a long period of latency between the time of inoculation and the appearance of a demonstrable tumor. But this possibility does not account for all failures because, while some investigators either omit information concerning the time their animals were under observation, others (*5*, *10*) kept the inoculated mice for as long as 15 months and noted that the implanted tumors failed to grow. Such evidence suggests that nongenetic factors exert some influence upon the ability of transplanted primary hepatomas to grow in genetically susceptible hosts.

The inability of 43 primary hepatomas to grow in any member of their strains of origin suggests that the tumors were non-neoplastic in the usual sense of the word. The nature of primary hepatomas was discussed previously (*5*) and the reasons for interpreting them as neoplastic or regenerative were summarized. It was concluded that while the evidence favored their neoplastic nature it was not possible to state whether they were benign or malignant because metastases are rare and transplantability is not a criterion of malignancy.

Transplantation experiments have shed little light on the nature of the lesions. Of the 15 tumors that grew when transplanted, 7 were called "liver masses" and no histologic diagnoses were given (*7*), 4 were diagnosed as "hepatomas" (*3*, *5*, *8*, *10*), 1 as a "benign hepatoma showing adenomatous characteristics" (*1*), 2 as "liver-cell carcinomas" (*6*) and

1 as "malignant" (*9*). The "malignant" tumor grew in but 1 of 8 inoculated mice, and another tumor diagnosed as "malignant" failed to grow in 13 recipients (*9*). Three other tumors diagnosed as liver-cell carcinomas (*6*) also failed to take in inoculated mice. Briefly, the literature contains reports of the inoculation of inbred mice with a benign hepatoma that grew, whereas transplants of malignant liver-cell tumors and liver-cell carcinomas did not grow. Obviously, the relationship between the histologic appearance of primary hepatomas and their ability to propagate in new hosts deserves more consideration.

This report deals with the transplantation of primary induced or spontaneous hepatomas in inbred mice to ascertain 1) whether nongenetic influences are involved in their abilities to undergo propagation in their strains of origin, and 2) whether animal passage of the tumors would supply evidence of their benign or malignant nature.

The first objective involved the transplantation of primary hepatomas into a small number of mice and keeping the inoculated animals under observation for a considerable period of time. If, under these conditions, all recipients developed tumors, genetic factors would be of prime importance in the propagation of hepatomas. If, as in previous studies, not all recipients developed tumors, then nongenetic influences could be involved and efforts could be made to ascertain the nature of such influences.

The second objective was dependent upon histologic studies of primary and transplanted hepatomas and included two approaches. The first consisted of an attempt to correlate the histologic structure of primary hepatomas with their abilities to propagate in new hosts. The second, which was the chief aim of the investigations, consisted of histologic studies of serial passages of the tumors to ascertain whether the primary hepatomas showed any change in morphology correlated with an increased growth rate. This phase of the investigation was based upon the assumption that the relatively slow growth rate of many primary hepatomas is to be expected in view of their slow rate of development in older animals but, if permitted to evolve further in new hosts, they would assume the biological properties generally ascribed to malignant tumors. It is conceivable that the tumors start as a hyperplastic process which later develops into a true neoplasm.

The results indicated that many primary hepatomas are propagable when 3 or 4 mice of their strain of origin are used as recipients for the first-passage generation. Most of these transplanted primary hepatomas were carried through serial passages. The majority retained the microscopic characteristics of the primary tumors, but some underwent a pronounced change in histologic structure.

MATERIALS AND METHODS

The study included 4 experiments extending over a period of 13 years; the first was started at the Harvard Medical School, Boston, Mass., and

was completed at the National Cancer Institute, Bethesda, Md.; the remaining 3 were carried out within the laboratories of the National Cancer Institute.

All mice used in these investigations were from inbred strains C, I, or C3H (*11*) which were raised in this laboratory. They were fed a diet of Purina dog chow or laboratory chow and had access to tap water at all times. The diet was not changed during the course of an experiment. Mice inoculated with primary tumors were 2 to 3 months of age; those used for serial passage were from 2 to 6 months old. Both sexes were used as recipients and showed no obvious sex difference in ability to propagate the transplanted tissues. Only nonbreeding mice were employed.

Induced tumors were procured by the administration of *o*-aminoazotoluene, 3:4:5:6-dibenzcarbazole or carbon tetrachloride. The amount administered is given in the description of individual experiments.

The *o*-aminoazotoluene was obtained from the Eastman Kodak Co. and was used without purification. Shear's technique of administration (*6*) was used: the crystalline compound was moistened with glycerol (6 drops to each 200 mg.) and injected subcutaneously into the right axilla by means of a 12-gauge trocar equipped with a snugly fitting plunger. The injections, consisting of approximately 10 mg. each, were repeated at monthly intervals.

The 3:4:5:6-dibenzcarbazole (m.p. 156.5° C. corr.) was procured from Professor J. W. Cook of Glasgow, Scotland. This compound was also administered subcutaneously in the right axillary region. Each mouse received a single injection of 0.2 mg. of the compound dissolved in 0.2 cc. of olive oil.

The carbon tetrachloride was of "C. P." quality. Four cc. were dissolved in 100 cc. of olive oil and a dose of 0.25 cc. of the mixture administered orally by means of a stomach tube. The injections were repeated at weekly intervals.

A uniform procedure was followed for the transplantation of all tumors. The primary hepatoma or transplanted hepatoma was removed, measured to determine its average diameter,[3] a note made of its gross appearance, and then cut into two pieces by means of a razor blade. One piece was prepared for histologic examination. The remainder (that part of the tumor immediately adjacent to the portion used for fixation) was used to inoculate mice of the strain in which the primary tumor or transplanted tumor had developed. The usual trocar technique was used: the tumor pieces were cut to fit snugly into a 12-gauge trocar and implanted into the subcutaneous tissues of the right axilla. The point of the trocar was inserted through the skin of the mid-dorsal region, passed forward and downward into the axilla, and retracted slightly before the plunger was pressed to expel the inoculum.

[3] Throughout this paper the diameter of a tumor is the arithmetical average of 3 measurements, namely, length, width, and thickness. A tumor 20 mm. long, 10 mm. wide and 6 mm. thick is designated as 12 mm. in diameter.

All tissues were fixed in Tellyesniczky's fluid (2 parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid), embedded in paraffin, and stained with hematoxylin and eosin. The periodic acid-Schiff technique was used on a representative number of sections, and the prussian-blue reaction was used on a few sections in which brown hemosiderin pigment was prominent in the hematoxylin and eosin sections. No tissue was preserved in nonlipoid solvent fluids, and no fat stains are available.

EXPERIMENTAL PROCEDURES AND GENERAL RESULTS

Four experiments yielded 36 primary tumors that were transplanted successfully or propagated through serial passages. The source of each tumor and results obtained with it are summarized in table 1. The seventh column of the table, designated as the transfer periods, includes the number of days elapsing between the time the primary tumor was implanted and the resultant growth used for the second passage, and the comparable time required between the last two transplant generations. The time between transfers is presented because the inoculated animals were not examined at regular intervals and, of more importance, it was difficult to determine precisely when a very slowly growing tumor was first detectable. The data reveal the long period of time required for the first-transplant generation of hepatomas to become established in new hosts.

It is also seen that the transplanted hepatomas acquired the ability to grow faster during serial passage. This finding was in harmony with those of other investigators (*2*, *5*, *6*, *8*, *9*), and showed that hepatomas resemble other transplantable tumors in this respect.

The last column of the table includes the number of mice used to propagate the tumor through all passages and the number of animals in which the tumor grew. Some tumors grew in all hosts, but others failed to become established in a significant number of animals. This latter information is not exact for all tumors because some tumor-free mice were killed when the laboratories were moved from Boston to Bethesda, and a few were killed accidentally. Despite these deaths there was sufficient evidence to show that all tumors did not grow in all the recipients. This outcome provided an answer to one of the purposes of the studies, namely, that nongenetic factors probably exert some influence in the transplantability of hepatomas.

General Histologic Observations

All the sections taken from these tumors were examined. A special search was made for: 1) any difference which might be found between spontaneous tumors and those induced by *o*-aminoazotoluene or carbon tetrachloride, 2) some morphologic difference between the tumors which were successfully propagated and those which failed to grow on transplantation, 3) some histologic feature which might be correlated with the growth rate of the different tumor lines and particularly with the changes

TABLE 1.—*Summary of findings with 36 successfully transplanted hepatomas in inbred mice*

Tumor number	Spontaneous or induced	Carcinogen used	Strain of origin	Sex of mouse	Number of transplant generations	Transfer periods		Passage mice	
						First generation (*days*)	Last generation (*days*)	Number inoculated	Number in which tumor grew
1	Induced.....	*o*-amino-azotoluene.	I	F	11	107	14	34	25
2*	"	"	I	F	6	107	47	16	11
3†	"	"	C	F	11	107	14	34	27
4†	"	"	C	F	8	107	31	26	19
5	"	"	I	F	8	163	54	27	26
6	"	"	I	F	8	208	86	27	21
7*†	"	"	C	F	2	225	20	9	3
8†	"	"	C	F	8	225	32	24	19
9	"	"	C3H	M	8	258	106	36	31
10†	"	"	C	F	15	62	42	45	45
11	"	"	C	M	6	126	69	17	17
12	"	"	C	M	7	149	68	21	20
13	"	"	C	M	9	162	56	23	21
14	"	"	C	M	6	179	92	16	16
15	"	"	C	F	5	186	126	13	12
16*	"	"	C	F	1	186	.....	4	1
17	"	"	C	M	8	247	40	23	22
18†	"	"	C	F	8	252	14	25	19
19	"	"	C	F	5	344	53	16	13
20	"	"	I	M	4	76	160	11	9
21	"	"	I	F	7	114	67	20	19
22*	"	"	I	F	3	132	130	13	7
23	"	"	I	F	5	203	49	15	12
24	spontaneous.	.....	C3H	M	5	240	60	.....	.....
25	"	.....	C3H	M	7	92	71	20	19
26	"	.....	C3H	M	6	100	75	17	12
27	"	.....	C3H	M	6	131	36	17	14
28	"	.....	C3H	M	6	306	42	12	12
29‡	"	.....	C3H	M	6	273	34	19	14
30§	"	.....	C3H	M	6	341	40	15	13
31‖	"	.....	C3H	M	6	328	21	16	15
32*¶	"	.....	C3H	M	1	337	.....	3	1
33	"	.....	C3H	M	4	362	99	9	7
34	"	.....	C3H	M	3	341	120	9	7
35	"	.....	C3H	M	2	375	245	5	4
36	"	.....	C3H	M	3	350	225	6	4

* Failed to undergo serial transplantation.
† Transformed to hemangioendothelioma.
‡ Transformed to osteogenic sarcoma.
§ Transformed to cholangioma.
‖ Transformed to reticulum-cell sarcoma.
¶ Transformed to unidentified epithelial neoplasm.

in growth rate appearing in different generations, and 4) any histologic change preceding or accompanying a profound change in morphology, such as occurred in a number of the hepatomas.

With these purposes in mind, many different features of the tumor sections were investigated. These are described below and the variations in these features, as applied to hepatomas in general, are discussed. Histologic observations on particular tumor lines are given in the text after descriptions of the biologic behavior and gross appearance of each line.

1. Degree of resemblance to normal liver

The histology of these hepatomas was, in general, remarkably uniform and rather closely reproduced the structure of the normal liver (fig. 1). They were usually composed of cords of cells not more than one or two cell layers in thickness, which were separated by sinusoids lined by flattened endothelial cells. The sinusoids contained a variable amount of blood; often they were empty and the hepatoma cells were so closely apposed that they appeared as solid nests of cells with some thin strands of endothelial tissue separating them. Kupffer cells and bile ducts were not found in transplanted tumors, and only at the margins of the primary tumors, where they had probably persisted from extension of the tumor into pre-existing normal tissue. A notable difference in the hepatoma, in comparison with normal liver, was the development of focal areas of cells of a different morphology so that nests of greatly vacuolated cells, or nests of larger size with thick cordlike structure often appeared among the more uniform "background" cells of the hepatoma. There was no true lobule formation in the hepatomas though occasional tumors showed a pseudolobulation due to unequal vacuolation of the hepatoma cells (fig. 2). The hepatoma tissue usually appeared more basophilic after hematoxylin and eosin staining than did the normal liver. As a rule, there was no true capsule formation but the hepatoma often appeared to compress the normal liver cells (fig. 3).

2. Size of hepatoma cells

The size of the hepatoma cells showed considerable variation. Many of the spontaneous tumors were composed, especially at the margins, of cells having nuclei smaller than those of the normal liver, and a small amount of cytoplasm which was considerably more basophilic than that of normal cells (fig. 3). That cancer cells should have nuclei smaller than the nuclei of the normal cell of origin is remarkable, and contrary to one criterion often proposed for malignant tissue, namely an increase in cell size, particularly of the nucleus. Primary tumors composed of small cells appeared to be more frequent among the spontaneous tumors in C3H mice than among the tumors induced by *o*-aminoazotoluene, although no direct comparative measurements were made.[4] Nests of larger cells were frequently found within the hepatoma, which might compress the surrounding hepatoma tissue, much as the hepatoma had compressed the normal tissue, producing an effect of "a tumor within a tumor" (fig. 4). Occasional cells were found of giant size with large, bizarre nuclei. Focal areas made up entirely of large cells or entirely of small cells were usual, rather than a haphazard intermingling of cells of various sizes.

[4] A paper by Dr. Toru Miyaji (*12*) concerning the size variation in hepatoma cells will appear in this Journal. Some of the material included in Dr. Miyaji's study was obtained from transplanted spontaneous tumors from this series. He found that the volume of the nuclei of hepatoma cells showed roughly the same proportionate variation in size, that is in a 1:2:4:8 ratio, as do normal liver-cell nuclei, and the differences in size are not haphazard.

3. Degree of vacuolation of cytoplasm

The degree of vacuolation of the cytoplasm varied greatly, and was occasionally so extreme that the hepatoma tissue histologically resembled adipose tissue (fig. 5). Cells with a similar degree of vacuolation of cytoplasm were usually found in the same area, often forming nests of vacuolated cells. The nature of the substance within the vacuoles was not determined. The periodic acid-Schiff reaction showed a few fuchsinophilic granules, which appeared to be insufficient to fill the vacuoles. No fat stains were done, but previous studies by Edwards and Dalton (*5*) on one of these tumor lines demonstrated a considerable amount of fat in early generations. Glycogen as demonstrated by Best's carmine stain was also abundant, and large vacuoles were sometimes seen which did not give a reaction for either fat or glycogen, and it was suggested by these authors that this was an hydropic change. This vacuolation did not appear to be a necrobiotic change in our material for it was not associated with areas of necrosis.

4. Basophilic granules or rods in the cytoplasm

Basophilic material which probably represents pentosenucleic acid was very prominent in some tumor cells, appearing as rings of finely granular or filamentous material enclosing clearer areas of cytoplasm (fig. 6). The nucleus usually appeared in the periphery of this ring. This basophilic substance appeared to be more prominent in many of the tumors with a rapid growth rate, but there was no regular correlation of the amount of this substance and growth rate. A similar basophilia appears in the human liver, and has been the subject of several recent reports (*13*). It is to be found in the normal mouse liver, but appears with unusual distinctness in some neoplastic cells.

5. Cystic spaces in the tumor

Cystic spaces were frequent in the hepatomas, especially in the transplanted tumors. These spaces sometimes appeared to be lined directly by liver cells, and often contained a fine granular eosinophilic substance (fig. 7). The histogenesis of these cysts was uncertain. It was probably not due to necrosis, since the marginal cells appeared healthy. Some of these cysts were filled with blood, which was probably due to secondary hemorrhage. Cells showing a positive prussian-blue reaction were found in the walls of these cysts, and some of the cysts were undergoing organization (fig. 8). Such repair processes have been reported for other neoplasms. These cysts often occupied considerable space, and may have so increased the bulk of the tumor as to give a false impression of the growth rate in many cases.

6. Formation of cords of tumor cells and separation of these cords by blood-filled sinusoids, or fusiform cells

Many of the tumors, especially after transplantation, became very vascular, and histologic examination showed cords of hepatoma cells of varying size separated by blood-filled sinusoids. This feature was particularly observed and noted in the histologic examination of the tumors, since it seemed possible that such a development was preliminary to the formation of hemangioendothelioma, which developed in many tumor lines, but no correlation to this process was found in the "transformed" lines. Such highly vascular areas are found in other transplanted tumors, notably mammary tumors, and probably result from the disproportionate growth of tumor tissue and nutrient vessels.

Some of the induced tumors, in particular, showed a proliferation of fusiform cells in the sinusoidal spaces between the hepatoma cords. These may have originated from endothelial cells, and suggest a proliferative capacity of such cells within the tumor. They merit consideration because of the development of hemangioendotheliomas on transplantation in some of the induced tumors, but again this formation was not observed more frequently in "transformed" lines (fig. 9).

7. Cytoplasmic inclusion bodies

Hyaline bodies were frequently observed in the cytoplasm of the tumor cells (fig. 10). These bodies have been described before in connection with hepatomas in the mouse (*4*) and in nontumorous livers. In nontumorous livers they may appear in such numbers as to produce lesions which are grossly visible and may be mistaken for tumors. Unsuccessful attempts at transferring liver tissue containing these bodies have been reported by Campbell (*14*). He also described histochemical studies of these formations, and noted that they are distinct from bodies with a finer granulation which resemble the inclusions in infectious ectromelia. In the present material where large single bodies were found in most cells in an area, cells in adjacent areas often showed a variety of other cytoplasmic changes. These altered cells might contain a large vacuole within which was finely granular hyaline material, or cells might be found with many small vacuoles, or numerous small granules. The large single bodies often appear to be molded and frequently show a central depression. The substance composing them appears to have a waxlike consistency, so they have been referred to in this laboratory as "waxy" bodies to distinguish them from other hyaline bodies. These bodies, and the frequently accompanying alterations in the cytoplasm of adajcent cells, appeared to be slightly more frequent in the tumors which did not grow, but no absolute correlation to biologic behavior could be made. These cytoplasmic bodies in the liver of the mouse are unlike the "Mallory bodies" (*15*), which are found in human cirrhosis of the liver. The "Mallory bodies" do not appear to have the molded waxlike consistency, and are less sharply outlined than the structures in the mouse.

8. Intranuclear inclusion bodies

Intranuclear inclusion bodies have previously been described in the liver cells of apparently normal mice. It was the opinion of Findlay (*16*), who did some experimental work with them, that they were caused by a filterable virus of low pathogenicity. He found them to be especially frequent in mice obtained from one source, and he gave detailed descriptions of them. Andrew *et al.* (*17*) have also described them in the livers of mice and of men, and correlate them with increasing age. Similar inclusions were not infrequent in the liver cells of the tumors. They may be an indication of the long growth period in these tumors.

9. Extramedullary hematopoieses

Evidence of extramedullary hematopoieses were not infrequent, and in one transplanted tumor line this reaction occasionally appeared to an extreme degree. The newly forming cells were of the erythrocytic series, and small clumps of nucleated red cells could be found within the sinusoids of the tumor (fig. 11). Occasionally, single or many megakaryocytes were found (fig. 12). Apparently conditions in the tumor furnish the necessary stimulus and environment for the formation of these two cell types.

10. Mitotic figures

A comparison was attempted between the number of mitotic figures and growth rate. All mitotic figures from 10 high-power fields (× 300) of a number of selected sections were counted. Ten transplanted lines were chosen and the number of mitotic figures from the generation having the longest period of growth was compared with the generation having the shortest period of growth. The results were:

Generation with longest growth period (150–343 days' range)—
Average number of mitotic figures=14.
Generation with shortest growth period (36–70 days' range)—
Average number of mitotic figures=33.

Another comparison was made between three generations of 2 hepatoma lines having a generally slow growth rate, and three generations of 2 other lines with a fast rate as indicated by tumor size. The results were:

Hepatoma 36 average growth period 251 days—mitotic figures=5
Hepatoma 15 average growth period 149 days—mitotic figures=2
Hepatoma 21 average growth period 48 days—mitotic figures=17
Hepatoma 13 average growth period 48 days—mitotic figures=34.

There thus appears to be a rough correlation between growth rate and mitotic figures. Certain striking inconsistencies were observed, however. In hepatoma 6, the first generation of 208 days contained 8 mitoses, while the sixth generation of 48 days contained only 7, and in hepatoma 13 the first generation of 162 days had 26 figures, while the fifth generation of 40 days had only 15. The largest number of mitotic figures, 93, was present in the seventh generation of hepatoma 17, which had the shortest growth period of all, 36 days. There thus appears to be a higher number

of mitotic figures in rapidly growing than in slowly growing tumors, and estimations of malignancy made from the number of mitotic figures in biopsy specimens may be worth undertaking. The inconsistent results may be due to our inability to measure the rate of growth at the precise time the count is made and the difficulty of measuring the absolute size of a tumor, since differences in the diameter do not always represent actual increase in bulk of tumor tissue. Casey (*18*) has made mitotic counts of human neoplasms and reported a correlation between malignancy and number of mitoses.

11. Results of the periodic acid-Schiff technique

This was done on a selected group of slides which consisted of a number of primary and random transplanted tumors, and the complete series of transplanted hepatoma lines 2, 13, 30, and 36. The periodic acid-Schiff-positive material present in the liver is presumed to be largely glycogen, and this reaction in the liver can be regarded as a histochemical test for glycogen, since other polysaccharides are of negligible importance in the liver cell. The periodic acid-Schiff reaction varies considerably in the livers of normal mice, in which glycogen may be abundant or practically absent. When present, however, it will be found uniformly distributed throughout the entire section, and it will have a regular arrangement in relation to the normal lobular architecture of the liver. In the hepatomas the amount of glycogen might be increased (figs. 13, 14) or decreased (fig. 15) in comparison to the adjacent nontumorous liver, or it might be about equal in amount. In three of the hepatoma lines (Nos. 2, 30, and 36) the glycogen content of the primary tumor was high, and all the transplants showed a considerable amount of glycogen. In the fourth line studied (No. 13), the glycogen content of the primary tumor was similar to that of the adjacent liver, and in general the transplants showed small amounts, although fluctuations occurred which could not be correlated with growth rate. The most striking difference between the glycogen distribution of the normal liver and that of the hepatoma, however, was that no relation of the content of glycogen to architecture was found in the hepatomas. Indeed, a great difference in the quantity of glycogen was sometimes observed in contiguous cells, although no other morphologic differences were apparent (fig. 16). Hepatoma cells in which glycogen was present were sometimes found in mitosis. It appears from this that mitosis in a hepatoma cell is not incompatible with the presence of glycogen, and a rapidly proliferating tumor may contain a high content of glycogen. The variability of the amount and distribution of the histochemically demonstrated glycogen is in accord with the variable results obtained after chemical studies of the alkaline and acid phosphatase of hepatomas in rats (*19*), and suggests the difficulty of demonstrating a quantitative chemical difference between normal and neoplastic tissue, since individual cells may differ so greatly.

12. Condition of the nontumorous liver

The nontumorous areas of the livers adjacent to the primary hepatomas usually appeared normal in the C3H mice with spontaneous tumors. The non-neoplastic liver of mice which had received *o*-aminoazotoluene or carbon tetrachloride showed the changes described for these agents (*10, 20*). There were occasional cystic areas lined by cuboidal epithelium, apparently developed from bile ducts. Minute areas with abnormal cells were often found in the normal tissue adjacent to the main tumor mass, especially with the induced tumors, and probably represented multicentric foci of neoplastic growth.

RESULTS IN INDIVIDUAL EXPERIMENTS

Experiment 1

The first 9 tumors of the table were obtained in the first experiment and 7 were mentioned briefly in an earlier publication (*7*). Hepatomas were induced by the administration of 11 injections (totaling approximately 110 mg.) of crystalline *o*-aminoazotoluene.

Thirty primary tumors from 19 mice were each transplanted into 1 or 2 mice; 20 were used to inoculate 1 mouse and 10 to inoculate 2 mice. Of the 9 transplantable hepatomas procured, 6 (Nos. 1, 2, 3, 4, 6 and 9) were derived from the inoculation of 1 mouse and 3 from the inoculation of 2. Of the latter, one (No. 5) grew in both recipients and 2 (Nos. 7 and 8) grew in but one; both tumor-free mice survived for 11 months after inoculation. These results suggest that the use of more than one recipient for 20 of the primary growths may have increased the number of successes.

Of the 19 donor mice, 9 each supplied 2 primary hepatomas and from these 18 tumors 6 of the propagable growths were obtained. Of these 6 tumors, 2 (Nos. 7 and 8) were derived from the same mouse and 4 (Nos. 2, 3, 4 and 6) were derived from different animals. Since these 4 mice also supplied 4 tumors that did not grow, it is clear that the ability of hepatomas to propagate in new hosts was not dependent upon the mouse in which they occurred.

Of the 30 transplanted primary hepatomas, 10 arose in strain C females and 4 (Nos. 3, 4, 7 and 8) were propagable, 9 arose in strain I females and 4 (Nos. 1, 2, 5 and 6) were propagable, 4 arose in strain C3H males and 1 (No. 9) was propagable, 7 arose in strain I males and none grew.

Recipient mice for the 21 tumors that failed to grow were kept under observation from 7 to 11 months after inoculation. Pertinent details of the 9 propagable hepatomas derived from this experiment are presented below. Many of the descriptive terms were taken from the notes made at the time of transplantation.

Hepatoma 1 arose as "a liver-colored mass 10 mm. in diameter" and retained its macroscopic appearance throughout all animal passages. Its transfer period gradually decreased until the seventh passage, when it became stabilized at 14 days. One mouse was used for the first passage. Of 3 mice inoculated for the second-transplant generation, 2 developed tumors within 2 months and 1 within 5 months. One of the

earlier tumors was used to inoculate 5 mice for the third passage. Three developed tumors within 2 months and 2 died tumor-free 8 months after inoculation. Up to the seventh passage the tumor failed to grow in an occasional mouse but thereafter it grew progressively in virtually all strain I mice. Five strain Y mice were inoculated from a tenth-passage tumor and all were resistant. This tumor was relatively easy to carry in strain I mice and was discontinued after undergoing 27 passages.

Microscopically, the primary tumor closely resembled normal liver, and was not clearly demarcated from it. Size of the nuclei in the tumor varied considerably, a condition also present in normal liver (*12*). One focus of greatly vacuolated cells was found within the tumor. Occasional well-developed "waxy" bodies were present and cells within the same area showed large vacuoles containing a granular eosinophilic substance, or numerous hyaline bodies of rather uniform size. The adjacent normal liver showed an area of small cystic spaces, lined by low cuboidal and flattened cells. These lining cells were more cuboidal than is usually seen with endothelial cells, and these spaces did not contain erythrocytes.

The first-generation transplant still closely resembled normal liver, but the cells were larger and less uniform in size. Large hyaline bodies were found in occasional cells. Areas of the tumor showed necrosis and considerable infiltration with granulocytes.

In the second generation there was an increased amount of fibrous tissue, much of it appearing as single fusiform cells between cords of liver cells. One large cystic area containing erythrocytes was present in the section. The third and fourth generation showed a moderate increase in fibrosis, and there was an increase in the cystic spaces, many of which contained erythrocytes. Hepatoma cells lining these spaces were observed to be larger in size than surrounding hepatoma cells. Hyaline cytoplasmic inclusions were not found in later generations, and areas were frequent in which cords of liver cells were widely separated by vascular spaces.

Hepatoma 2 was described as "liver-colored, 18 mm. in diameter" and retained its original macroscopic appearance throughout all 6 transfers. One mouse was used for the first transplant, but the tumor did not grow in all inoculated animals of succeeding generations. Two mice were used for the sixth passage and, 47 days later, one had a tumor which was 11 mm. in diameter. This tumor was used to inoculate 2 mice, both of which died without tumor 6 months later. Hence, it is listed in the table as a tumor which failed to undergo serial transplantation.

The primary tumor resembled normal liver, but one area showed extreme vacuolation of the hepatoma cells. Vacuolation of cells in this tumor gradually increased during the transfer generations, and the sixth showed an extreme degree of vacuolation which affected nearly all the cells in section. It is possible that such vacuolated cells are less viable on transplantation.

Hepatoma 3 arose as a "liver-colored" tumor 10 mm. in diameter and maintained its appearance after remaining within the first-passage mouse for 107 days. From the second passage on, the gross appearance of the tumor was "gelatinous," "hemorrhagic," "soft and bloody," usually surrounded by a "bloody exudate" or by "a thin layer of tissue." The tumor was easy to propagate and was discontinued after the eleventh passage.

The primary tumor resembled normal liver, but the cells were larger, and binucleate cells and cells with large, irregular bizarre nuclei were frequent. An area of slightly different structure, or "tumor within tumor," was observed in which cords of large hepatoma cells were separated by vascular spaces. The first generation resembled the primary, but the section from the second generation was a hemangioendothelioma, with no evidence of hepatoma structure (fig. 17). In succeeding generations this structure was maintained and sections often showed no more than a thin cyst wall containing blood, lined by neoplastic cells with an outer capsule of granulation tissue such as appeared in several transplanted lines (fig. 18). In some sections, the wall of the cyst was composed entirely of granulation tissue and no neoplastic cells were found. It is understandable why transplants from such tissue might fail to grow due

to the absence of viable tumor cells. One feature observed in this hemangioendothelioma on transplantation, which has been reported previously (*21*), was a zone of congestion extending into the surrounding adipose tissue, at some distance from the actual neoplastic cells (fig. 19).

Hepatoma 4 originated as a "white firm mass 4 mm. in diameter" and was grafted into 1 mouse that, 107 days later, had a tumor 14 mm. in diameter which was "pale and firm." This tumor was implanted into 4 hosts of which 2 had palpable tumors within 2 months and 2 died tumor-free 7 months after inoculation. The transplanted tumor (second generation) was described grossly as "excellent tissue with appearance of liver." The gross descriptions of this tumor are of interest. For the third generation it "resembled liver," for the fourth, "solid and color of liver," for the fifth "very hemorrhagic" (this passage grew in only 1 of 3 mice), and for the sixth "a cyst full of blood." Pieces of the cyst wall were used for inoculation and the seventh passage was also described as "a cyst full of blood." The tumor was discontinued after 8 passages.

The primary tumor resembled liver, but there was a focus with a more obvious cord formation, separated by vascular spaces, and another focus of much larger cells. The first generation was similar, except that areas of a more glandular structure were found than is often encountered in hepatomas in the mouse, and rows of hepatoma cells encircled small lumenlike spaces. This structure continued through the next two generations, with a slight increase of fibrous tissue. In the fifth generation, section of the transplant showed a wall of granulation tissue containing erythrocytes and blood clot. No hemangioendothelioma cells were found. (Sections from tumors of this type occasionally fail to include the neoplastic cells.) The sixth, seventh, and eighth generations showed a narrow layer of neoplastic hemangioendothelioma cells, often only 1 cell thick, surrounding blood-filled spaces. The hemangioendothelioma cells sometimes appeared to be phagocytizing erythrocytes. Considerable lymphocytic infiltration appeared in the last generation.

Hepatoma 5 arose as a "white firm mass 14 mm. in diameter" and was easily propagated through 8 serial passages. Despite a transfer period of approximately 60 days for the last 5 transfers, and in contrast to the more rapidly growing hepatomas 1 and 3, it grew in virtually all inoculated mice. The first-transplant-generation tumor was "the color of liver" and all subsequent generations were similar. This tumor was discontinued after 8 transplant generations. Histologically the original tumor resembled normal liver. No remarkable change developed during transplantation.

Hepatoma 6 was started from a "liver-colored" tumor 17 mm. in diameter. The inoculated mouse had a small palpable mass at the site of implantation within 180 days, and when transplanted 28 days later it was 11 mm. in diameter. Only 1 of 5 mice of the second generation developed a tumor, which was 10 mm. in diameter 4 months after inoculation. All subsequent passages of the tumor grew slowly in virtually all mice and maintained the gross appearance of the primary tumor. It was discontinued after 8 passages.

The primary growth resembled normal liver, except that cells contained more cytoplasm, although nuclei were of similar size. All passages of this tumor resembled normal liver, with varying degrees of vacuolation of cells. Cystic areas developed in some generations, and there was a variable amount of fibrosis, sometimes with apparent calcification.

Hepatoma 7 was of unusual interest. The primary tumor was 10 mm. in diameter and "liver color" in appearance. Tumor grafts were implanted into 2 mice; one did not develop a tumor and died 10 months following inoculation, the other had a tumor 14 mm. in diameter after a transfer period of 225 days. This tumor was very hemorrhagic and was used to inoculate 4 mice. Two of the second-passage-generation mice died tumor free 6 months after inoculation but the other 2 had palpable masses at the inoculation site within 19 days after inoculation. One was killed the following day when its tumor measured 13 mm. in diameter, and was described as "cystic and

full of blood." Pieces of the cyst wall were implanted into 3 mice that died tumor-free 6 months later. This tumor was placed among those that failed to propagate.

Cells of the primary tumor had an unusually large amount of cytoplasm, and waxy bodies were frequent. One focus showed cord formation in tumor cells, separated by wide vascular channels. Section of the first-generation tumor showed a hemangioendothelioma, which was similar in the second generation.

Hepatoma 8 originated in the same mouse that produced Hepatoma 7. The primary tumor was 16 mm. in diameter and hemorrhagic in appearance. It was grafted into 2 mice, one of which was positive and the other negative 225 days later. The transplanted growth was 18 mm. in diameter and very hemorrhagic. The tumor was propagated through 8 passages and retained its spongy hemorrhagic appearance. It was discontinued after 8 passages.

The primary tumor resembled normal liver but had areas in which the hepatoma cells were arranged in cords separated by vascular spaces. Section from the first generation showed only blood clot. Sections from succeeding generations showed only small numbers of hemangioendothelioma cells within the granulation tissue wall of a blood-filled cyst.

Hepatoma 9 occurred in a C3H mouse and was 9 mm. in diameter. It was implanted into one animal that showed no sign of tumor growth until 7 months later. The tumor grew very slowly and was only 11 mm. in diameter 258 days following inoculation. The primary growth and all passage tumors resembled liver grossly. Slow growth was the chief characteristic of this tumor; approximately 3 months elapsed before a palpable mass was present in any passage. After 8 such transfers the tumor was discontinued.

Histologically the primary tumor resembled liver. There were a few small areas with hepatoma cells in cords, separated by vascular spaces. Section of the first transplant showed a notable number of intranuclear inclusions, and basophilic substance in the cytoplasm was conspicuous. Nuclear inclusions and basophilic substance became less prominent in succeeding generations. In the seventh generation, the cytoplasm was noted to be more vacuolated, and many large blood-filled cysts had developed.

In this experiment 30 primary induced liver tumors were each implanted into 1 or 2 recipients. Nine tumors grew in their hosts and were transplanted from 107 to 258 days after inoculation. Seven were successfully propagated through from 8 to 11 serial passages; one failed to grow after 2 passages and one after 6 passages. Four became transformed to hemangioendothelioma.

In addition to the tumors described above, the 4 primary hepatomas transplanted by Emmart (*8*) arose in strain C3H males of this experiment and one (hepatoma E) grew in serial passage. The propagable growths derived from this experiment were used by various members of the staff of the National Cancer Institute. A communication from Greenstein *et al.* (*22*) contained a preliminary description of hepatoma 1 of this report and of hepatoma E (designated as A_4 in their paper) obtained from Emmart.

The 9 hepatomas derived from the first experiment have been described in considerable detail because they exhibited variables encountered in most other tumors of succeeding experiments and are, therefore, offered as prototypes. In the remaining 3 experiments, only those tumors which were of special interest will receive consideration.

Experiment 2

The second experiment included mice of strain C and was performed to ascertain the effect of castration and hormonal stimulation upon the induction of hepatomas (*21*). Each mouse received 4 injections of *o*-aminoazotoluene totaling approximately 40 mg. of the crystalline compound.

Thirteen primary induced hepatomas from 13 mice were transplanted of which 10 (Nos. 10 to 19, inclusive, of table 1) were propagable. Hepatomas 10, 15, and 19 were from intact females; hepatomas 16 and 18 from castrated females, and hepatomas 11, 12, 13, 14 and 17 from castrated males.

Of the 3 tumors that did not grow, one was from an intact female, one from a castrated female and one from a castrated male. All recipients of these tumors lived for 11 months following inoculation.

One primary tumor was grafted into 3 mice and all others into 2 mice each. Six grew in all recipients and 4 in 1 of 2 hosts. Resistant hosts of these 4 hepatomas (Nos. 16, 17, 18 and 19) were kept under observation for 11 months after inoculation. As seen in table 1, these 4 tumors grew slowly in their first-transplant-generation hosts. This suggests that the more resistant animals might have developed tumors had they lived longer.

Hepatoma 10 was implanted into 3 mice, all of which had palpable tumors within 60 days. It was the most rapidly growing tumor of the entire 36 listed in the table and grew in all inoculated mice. It has been used by investigators at the National Cancer Institute and has been sent to other laboratories under the designation of hepatoma 10. The tumor was discontinued in this laboratory after 15 animal passages.

The primary tumor resembled normal liver, though the cells were generally of larger size. Some cells were considerably vacuolated. Basophilic substance was prominent. There were occasional nuclear inclusions. The first-generation transplant resembled the primary, but no nuclear inclusions were found. Section of the second-generation transplant was of special interest, since it showed a hemangioendothelioma contiguous to the hepatoma (fig. 20) and invading muscle. The hepatoma resembled the primary. In the third generation, the section again showed hepatoma tissue only, as did the fourth, fifth, sixth, and seventh generations. No sections were available for the eighth, ninth, and tenth generations. Two tumors grew from the tenth-generation transplant, and both were hemangioendotheliomas. Two lines were continued until the fifteenth and fourteenth generations and all tumors were hemangioendotheliomas.

The sixth-generation transplant of this tumor was given to another investigator within the National Cancer Institute and is now in the twenty-eighth generation. It has continued to be morphologically a hepatoma. A second laboratory, which is located in New York City, also received this tumor when in the fourth-transplant generation. It continues to be a hepatoma.

Hepatoma 16 was described as a "liver-colored" tumor 10 mm. in diameter and was grafted into 2 mice. One mouse survived for 11 months and died free of tumor. The other developed a palpable mass at the site of inoculation within 100 days. The tumor grew very slowly and, 86 days later, when it was 12 mm. in diameter was used to inoculate 2 mice. Since both of these mice died without tumor 13 months later, the tumor is included in table 1 as one which was nonpropagable. Histologically the primary tumor resembled normal liver, and the first transplant was similar.

Hepatoma 18 was described as "a straw-colored mass 14 mm. in diameter" and was transplanted into 2 recipients. One did not grow the transplant within a period of

11 months. The other developed a tumor at the inoculation site 210 days following implantation. Forty-two days later it was described as "soft and hemorrhagic" and was transplanted into 3 mice. Forty days after implantation one animal had developed a tumor which was described as "a blood-filled cyst 20 mm. in diameter." Pieces of the cyst wall were implanted into other mice. The tumor continued to develop as a cyst full of blood and was discontinued after undergoing 8 passages.

The mouse in which hepatoma 18 originated had a subcutaneous hemangioendothelioma. The primary hepatoma resembled normal liver, but was remarkable for the considerable vacuolation of many groups of cells. Section of the first-generation transplant showed a central area of necrosis, surrounded by a heavy wall of granulation tissue. A focus of hemangioendothelioma tissue was present in this wall, and an unusual infiltration of adipose tissue by neoplastic cells was visible at the margin. Subsequent generations of this tumor showed a blood-filled cyst, surrounded by hemangioendothelioma cells and granulation tissue.

The remaining hepatomas (Nos. 11, 12, 13, 14, 15, 17, and 19) of this experiment were all carried through from 5 to 9 serial passages and then discontinued. All were easy to propagate and grew in virtually all recipients. Hepatoma 15 was of interest for it required approximately 120 days to become 10 mm. in diameter.

Hepatoma 11, in general, resembled other hepatomas reported. It was remarkable, however, in having a blood-filled cyst develop at the margin in the fourth generation, and a few vessels at the margin showed unusually large cells in the wall which might be neoplastic. The next two generations appeared as usual hepatoma.

Hepatomas 12, 13, 14, 15, 17, and 19 all resembled normal liver histologically and showed no remarkable alterations. Hepatoma 13 generally showed very prominent basophilic substance, and this was generally a rapidly growing tumor. Hepatoma 14 developed many cystic areas, many containing blood, in the sixth generation. In hepatoma 15, the primary tumor showed several unusual cell structures, where cuboidal hepatoma cells surrounded one to several greatly vacuolated cells (fig. 21). This particular structure was not seen in any other tumor. Transplant generations of this tumor generally resembled liver tissue, but some foci of very large cells were noted.

In this experiment, 13 primary induced liver tumors were each used to inoculate 2 or 3 mice. Ten became established in at least 1 host and 9 were propagated through from 5 to 15 serial passages. One failed to grow after the first-transplant generation. Two became transformed to hemangioendothelioma.

Experiment 3

Details of this investigation have not been published. Hepatomas were induced by the administration of *o*-aminoazotoluene, 3:4:5:6-dibenzcarbazole,[5] or carbon tetrachloride to strain I mice that were 3 to 9 months old when the experiment was started. Six subcutaneous injections of crystalline *o*-aminoazotoluene (totaling approximately 60 mg.), one subcutaneous injection of 0.2 mg. dibenzcarbazole dissolved in 0.2 cc of olive oil, and 22 weekly oral injections of 0.25 cc. of a 4.0 percent solution of carbon tetrachloride in olive oil were administered.

Results of transplantation are reported here because a number of primary hepatomas failed to grow in first-generation hosts. Nineteen tumors from as many mice were transplanted and only 4 became established within an observation period of 10 months. Three hepatomas induced with dibenzcarbazole were implanted into 1, 3, and 3 mice, respectively.

[5] Hereafter referred to as dibenzcarbazole.

None of the inoculated animals developed a tumor. Eight hepatomas induced with carbon tetrachloride were grafted into a total of 26 mice (6 into 3, and 2 into 4 animals) and none of the recipients developed a tumor. Eight hepatomas induced with *o*-aminoazotoluene were transplanted and 4 (Nos. 20, 21, 22, and 23 of table 1) were propagated successfully. Each of the 4 hepatomas that failed to grow was used to inoculate 3 mice.

Sections of the tumors in this experiment which failed to grow were examined, but no consistent morphologic difference was found which might account for the failure to transplant successfully.

Hepatoma 20 was the only tumor of the entire 36 which grew faster in the first-transplant generation than in subsequent generations. The primary growth was implanted into 3 mice all of which had palpable masses 62 days later. Two mice grew the tumor progressively and died within 150 and 180 days following implantation. The other mouse was killed 76 days after inoculation and had a tumor 10 mm. in diameter which was described as "encapsulated, adherent to skin and color of muscle." This first-passage tumor was implanted into 3 mice; palpable masses were apparent in 2 within 113 days and 37 days later one (14 mm. in diameter) was used for passage into 2 mice. It was described as "resembling liver." The third-passage tumor required 148 days to become 12 mm. in diameter and the fourth passage required 160 days to become 8 mm. in diameter. The transplantation was then discontinued.

Microscopic examination of this hepatoma showed that the primary growth resembled normal liver, but areas of small, elongated spindle cells appeared in the second-transplant generation. This may have accounted for its slower growth in later generations.

Hepatoma 22 was propagated successfully through 3 passages only and did not acquire any greater growth power. It grew in about half the number of mice inoculated in the first 3 generations and 3 mice used for the fourth passage failed to grow the tumor within 8 months following inoculation. It was regarded as a tumor that could not be carried through serial passages.

The other two hepatomas (Nos. 21 and 23) were relatively easy to propagate and both were discontinued.

Microscopic examination of hepatomas 21, 22, and 23 revealed that they generally resembled normal liver, and showed no remarkable alterations. Hepatoma 21 showed many cystic spaces, some filled with blood. That normal processes of regeneration can occur in neoplasms, was shown by the organization of the hemorrhages in some of these cysts, with a new growth of fibrous tissue (fig. 8). Basophilic substance was especially prominent in hepatoma 23.

In this experiment 3 primary liver tumors induced with dibenzcarbazole, 8 induced with carbon tetrachloride, and 8 induced with *o*-aminoazotoluene were each grafted into from 1 to 4 hosts. Only 4 of the *o*-aminoazotoluene tumors developed in the inoculated animals. Three were propagated through 4 to 7 serial transfers before they were discontinued: one failed to grow in animals of the fourth-transplant generation. No transformation of hepatomas was seen in this experiment.

Experiment 4

In this experiment, primary spontaneous hepatomas from strain C3H males were transplanted. With the exception of one (hepatoma 24), all were obtained from strain C3H males included in a recent report (*23*).

Hepatoma 24 arose in a strain C3H mouse of this laboratory and was designated by Edwards, Dalton and Andervont (*5*) as hepatoma 98/15

because, at that time, the mouse bearing the primary hepatoma represented autopsy No. 15 of experiment No. 98. The authors noted globular bodies within the cytoplasm of the liver cells, which were usually acidophilic ("waxy" bodies). The transplants were essentially similar to the primary tumor, but contained more mitotic figures. Fat stains made on fresh tissue showed a considerable amount of fat, and glycogen was abundant in the cytoplasm in all generations. Little or no stainable alkaline phosphatase was present. The globular bodies were regularly found in the transplants, often in large numbers. This tumor is still being carried within another laboratory at the National Cancer Institute and has undergone 64 transplant generations. Sections were reviewed, and showed no significant change from the original. Occasional generations showed a notable amount of erythropoiesis.

The remaining 12 transplantable hepatomas (Nos. 25 to 36 inclusive, of table 1) were obtained from 12 C3H males that were members of either the colony maintained in this laboratory or of a line deprived of the mouse mammary tumor agent (*24*). All the mice were 15 months old. Five of the primary hepatomas (Nos. 27, 32, 33, 34, 36) occurred in nonbreeding mice and 3 (Nos. 25, 29, 31) in castrates from the colony, while the remaining 4 (Nos. 26, 28, 30, 35) arose in nonbreeders from the agent-free line.

Of 6 primary hepatomas that failed to grow, 2 arose in nonbreeders and 2 in castrates from the colony; 2 arose in nonbreeders of the agent-free line. Two of these tumors were transplanted into 4 mice, 3 into 3 mice, and 1 into 2 mice. The 19 recipients were kept for 1 year after inoculation.

The spontaneous hepatomas in the C3H mice frequently appeared to be composed of cells smaller than the adjacent normal cells, and the cytoplasm appeared more basophilic (fig. 3). This was especially true of the tissue at the margin, while hepatoma cells in the interior were frequently larger and more eosinophilic, and the "tumor within tumor" appearance was frequent.

It is seen in table 1 that most of the 12 propagable spontaneous hepatomas required a long transfer period for the first-transplant generation and acquired an increased growth rate in subsequent passages. Eight (Nos. 25, 26, 27, 28, 33, 34, 35, 36) were implanted into a total of 20 mice for the first passage and grew in 13 of the recipients. All these tumors retained their gross characteristics and were successfully propagated through from 2 to 7 serial transfers before they were discontinued.

In these eight tumor lines the primary tumors were generally similar to each other in morphology, and the morphology and growth habits of the transplants were also similar. Hepatoma 25 developed a number of cystic spaces, and many nuclear inclusions were noted in the fourth and seventh generations in which the growth rate was slow. Hepatoma 27 was remarkable for the extreme vacuolation in areas of tumor cells.

Hepatoma 34 showed hyaline cytoplasmic inclusions in the primary tumor, and the growth rate was generally slow.

Hepatoma 29 was originally a "straw-colored mass 12 mm. in diameter." It was inoculated into 3 mice and grew in but one. At the end of 273 days in a new host it was described as "a partly straw- and partly liver-colored mass 9 mm. in diameter." This tumor was used to inoculate 4 mice and grew in one only. After a transfer period of 210 days it was a "soft, pale liver-colored mass 13 mm. in diameter." There were 3 recipients for the third passage and all developed tumors. Similar results attended all subsequent passages and the gross descriptions of the tumor were either "soft and straw-colored" or "pink and mushy." The tumor was discontinued after 6 transfers.

The primary tumor contained many greatly vacuolated cells. The first five generations were not notable except for the appearance of areas of greatly vacuolated cells, and the observation of many megakaryocytes within a small area in the second generation. Section of the sixth generation was entirely unlike hepatoma tissue, and consisted of cords of cells, in which the nuclei lay at the margin of the cords adjacent to cleftlike spaces while the center of the cords was composed of an amorphous eosinophilic material. The shape of the cells was a long oval, the nuclei were eccentric and located near the lumen, so that the cell cytoplasm seemed to merge into the amorphous substance toward the center of the cord. This central area was occasionally intensely eosinophilic, and areas suggestive of early ossification were present. Occasionally much larger cells were seen at the margin of the cords (fig. 22). This tumor was diagnosed as an osteogenic sarcoma because of its resemblance to such tumors previously observed in the mouse, in which central ossification is sometimes well advanced (*25*). Serial sections were made of the remaining paraffin block of this tumor, but no hepatoma tissue was recognized.

Hepatoma 30 arose as a "straw-colored mass 12 mm. in diameter" and grew in but 1 of 3 first-transplant-generation recipients. After a 341-day transfer period it was 15 mm. in diameter and described as "white and firm." Two mice were used for the second passage and both developed tumors within 40 days. The tumor retained its relatively rapid growth rate and a "firm, white and cystic" appearance in all subsequent passages.

Microscopically the primary tumor resembled normal liver, and included a considerable number of bile ducts at its margin (fig. 23). The first generation was entirely unlike hepatoma. Cores of coarse connective tissue were covered by large columnar epithelial-type cells and formed papillary projections into irregular spaces (fig. 24). There was considerable central necrosis in all transplants. This tumor did not resemble any familiar subcutaneous tumor of the mouse, and the fact that it retained the same uniform morphology in all transplants is contrary to the usual behavior of mammary tumors. The presence of proliferating bile ducts in the primary tumor may give a clue to its origin, for it is possible that this tissue, as well as the typical hepatoma, was neoplastic, and grew on transplantation. This may represent a cholangioma. No hepatoma tissue was found in the remaining paraffin block of the tumor, which was serially sectioned.

Hepatoma 31 was 14 mm. in diameter and described as "mixed straw- and liver-colored." It grew in 2 of 3 first-generation hosts and, 328 days after implantation, the largest tumor was 12 mm. in diameter, "cystic and full of blood." This tumor was grafted into 2 third-generation hosts and both had palpable tumors 14 days later. The tumor continued its rapid rate of growth for 6 generations and retained the appearance of "a firm, white cystic mass."

Microscopically the primary tumor was composed of small cells with greatly vacuolated, basophilic cytoplasm, and a central area of larger cells arranged in cords separated by wide vascular sinusoids. The first generation showed areas similar to hepatoma tissue and other areas appeared to be mixtures of spindle cells and epithelial cells. One area at the margin of the tumor showed the junction of tissue made up entirely of

spindle cells, and another type of tissue composed of hepatoma (fig. 25). The second generation appeared to be a fairly uniform mixture of nodules of hepatoma tissue separated by areas of spindle cells. The third, fourth, and fifth generation sections were generally similar to the second, but the amount of hepatoma tissue gradually decreased. The sixth generation was composed of fusiform cells resembling a fibrosarcoma, in which many cystic spaces occurred (fig. 26). Cells adjacent to these spaces had become rounded, many were detached and lying free. These detached cells were large and rounded, and contained one to several nuclei which were eccentrically located. Often there was a central area in the cell where the cytoplasm appeared clear and less eosinophilic. This tumor was carried to the twenty-sixth generation in another laboratory of the National Cancer Institute, and the two morphologic types of cells, rounded and fusiform, persisted (figs. 27A, 27B). The altered tumor was therefore diagnosed as a reticulum-cell sarcoma for these cell types are characteristic of this tumor in the mouse (*26*).

Hepatoma 32 was 10 mm. in diameter and straw colored. It grew in only 1 of 3 first-generation hosts. It was then implanted into 2 strain C3H females: one developed what appeared to be a mammary tumor and the other died tumor-free 180 days later.

Section of the primary tumor was typical of many of these spontaneous tumors. The cells were smaller than normal liver cells, and the cytoplasm more basophilic. The first generation was similar to the primary, except that the cells were even more basophilic. In the second generation, most of the tumor resembled an adenocarcinoma of an unusual type in which single rows of columnar cells formed a delicate papillary pattern. The centers of the papillae were composed of a delicate connective tissue. Near the margin of this tumor there was a mass of unmistakable hepatoma cells (figs. 28A, 28B), showing considerable fuchsinophilic substance with the periodic acid-Schiff technique. This hepatoma mass was completely surrounded by the adenocarcinoma.

This tumor was not transferred beyond the second generation because it was thought that the tumor arising in the C3H female was a spontaneous breast tumor common to this strain. Hence, the hepatoma was considered nonpropagable.

In this experiment, 19 spontaneous liver tumors from strain C3H males were each transplanted into from 2 to 4 hosts. Thirteen tumors developed in the inoculated animals from 92 to 375 days following inoculation and 11 were carried through from 2 to 7 serial passages before they were discontinued. One failed to appear in mice of the second-transplant generation. The remaining tumor, designated 98/15 in the literature, is being maintained in other laboratories.

Four of the transplanted hepatomas became transformed in new hosts: one to an osteogenic sarcoma, one to a questionable cholangioma, one to a reticulum-cell sarcoma and the last to an unidentified epithelial neoplasm.

GENERAL RESULTS

Transplantation study

In these studies, 23 primary hepatomas induced in strain C mice with *o*-aminoazotoluene were transplanted subcutaneously and 14 became established in the new hosts. Twelve tumors were propagated through from 5 to 15 serial passages and then discontinued, whereas 2 were lost after 1 and 2 transplant generations.

Twenty-four primary hepatomas induced in strain I mice with *o*-aminoazotoluene were implanted and 8 grew in the first-transplant generation.

Six underwent from 4 to 11 serial transfers before they were discontinued and 2 were lost after 3 and 6 passages.

Grafts of 3 primary tumors induced in strain I mice with dibenzcarbazole and 8 induced in the same strain with carbon tetrachloride did not grow in recipient mice.

Four primary liver tumors induced in strain C3H mice with *o*-aminoazotoluene were transplanted. One tumor grew through 8 transplant generations and was discontinued. Nineteen spontaneous hepatomas from strain C3H males were transplanted and 13 were found to be propagable. Eleven were carried through from 2 to 7 transplant generations and one did not grow in mice bearing the second-transplant generation.

Successful propagation of 19 hepatomas induced with *o*-aminoazotoluene and of 11 spontaneous hepatomas through serial passages established the feasibility of procuring transplantable liver tumors for experimental studies, especially when induced tumors from strain C females and spontaneous growths from strain C3H males are used.

Histologic review

After the conclusion of the histologic study, the results regarding the four points on which a special search was made can be given.

1) Comparison of spontaneous with induced tumors. Previous authors have considered whether morphologic differences could be detected between the following groups of liver tumors: spontaneous, carbon tetrachloride-induced (*10*), and *o*-aminoazotoluene-induced (*6*, *20*). Opinions expressed were that the tumors induced by *o*-aminoazotoluene could be diagnosed as "liver-cell carcinomas" [quoted from S. B. Wolbach in (*6*)]; those induced by carbon tetrachloride or *o*-aminoazotoluene appeared to be "well-differentiated hepatomas which resembled the spontaneous tumors of the mouse" (*10*), and none of the *o*-aminoazotoluene-induced "could be classified as adenocarcinomas" (*20*).

Comparison of the morphology of the variously derived tumors in the present material was attempted. The material was not satisfactory for such comparisons because of differences in the strains of mice. All but one of the tumors induced by *o*-aminoazotoluene which grew on transplantation were from strains C or I, whereas the spontaneous tumors which grew were all from strain C3H. Among the primary tumors which did not grow on transplantation there were 3 strain C3H tumors induced by *o*-aminoazotoluene, and 8 tumors induced in strain I by carbon tetrachloride. Four spontaneous tumors in strain I, and 6 tumors in strain C3H induced by *o*-aminoazotoluene, were also available from other sources. Before any sound conclusion can be reached regarding differences or similarities in the tumors, a much larger group of tumors from the same strains of mice must be available for comparison, and it is hoped that experiments which are now in progress will furnish such material. If actual differences exist they should be recognized under these conditions. The impression was gained from the present groups of tumors that the

cells and their arrangement showed a closer resemblance to normal liver in carbon tetrachloride-induced tumors in strain I, than did the cells and architecture of spontaneous tumors from other strains. These latter in turn appeared to be less anaplastic as a group than did the *o*-aminoazotoluene-induced neoplasms. This impression of a morphologic difference may be significant, since none of the carbon tetrachloride-induced tumors grew when transplanted. Most of these carbon tetrachloride-induced tumors were small in size and on microscopic examination they appeared as poorly demarcated collections of hepatic cells often distinguishable from normal liver only by the absence of Kupffer cells or bile ducts. These cell aggregates distorted the normal lobular architecture and sometimes compressed adjacent liver cells. Although most of the nodules were small, one was of considerable size (10 mm. in diameter) and it showed, as did the others, hepatic cells of a uniform size approximating the cells of the normal liver, with an orderly arrangement in cords (fig. 29). A slight cirrhosis and collections of pigmented "ceroid" cells noted by Edwards and Dalton (*10*) were seen in the non-neoplastic liver. Other foci of altered cells were usually found in these livers. These foci may have represented regenerative hyperplasia, or early neoplasms.

The spontaneous tumors in strain C3H were usually well demarcated from the adjacent liver, and the margin in particular was often composed of smaller than normal hepatic cells (fig. 3). Three of the tumors which attained a considerable size showed central areas of cells with large amounts of cytoplasm arranged in small clumps and separated by vascular sinuses (fig. 30). Such areas were morphologically very different from the structure of normal liver. The few spontaneous hepatomas available from strain I mice were remarkable for their large content of "waxy" bodies (fig. 10) which were not found in the adjacent normal liver. The marginal cells approximated the size of normal hepatic cells. The spontaneous tumors from all strains were usually single and they were often pedunculated.

Although many of the *o*-aminoazotoluene-induced tumors were similar to the spontaneous tumors, some showed an extreme variation in cell size even in a small area, and some tumor giant cells were found with multilobulated nuclei (fig. 31). These *o*-aminoazotoluene-induced tumors often appeared to develop from multiple foci, and the non-neoplastic liver showed the variation in cell size and evidence of cirrhosis (*20*) previously described. No differential characteristics between transplanted tumors grown from the induced or from the spontaneous primary tumors were recognized. It seems probable from these observations that if a difference exists in hepatic tumors which have arisen under different conditions in the mouse, the difference is one of degree, or the progression of neoplastic alteration, and not an identifiable qualitative distinction.

2) Possible morphologic differences between tumors which did not grow and those successfully propagated. Microscopic examination of the tumors which did not grow failed to reveal any consistent morphologic

difference in comparison with tumors which grew. Cytoplasmic hyaline inclusions (waxy bodies) and fibrosis were somewhat more frequently noted, and one primary tumor showed multiple small abscesses.

3) Possible histologic features which might be correlated with growth rate. None were found except for a high number of mitotic figures in some rapidly growing tumors.

4) Possible histologic change correlated with a profound change in morphology. No difference was found in the hepatomas which upon transplantation developed other types of tumors. Details of the transformation have been given in descriptions of different tumor lines.

DISCUSSION

The studies showed that any effort toward the establishment of transplantable liver tumors must take into account two biologic characteristics of primary hepatomas. The first of these is the long latent period following implantation of primary tumors into the tissues of genetically susceptible hosts. In the present experiments, the shortest period between implantation of a primary hepatoma and a resultant growth of approximately 10 mm. in diameter was 62 days, while the longest was 375 days.

The second characteristic is the failure of tumor transplants to grow in all inoculated mice, especially transplants from the primary tumors. The long latent period necessary for growth of implants from many primary tumors could be responsible for some failures but there is no reason to assume that all could be explained on this basis. If the long period of latency is accepted as a plausible reason for the nonappearance of tumors in every host, there remains the equally interesting problem of inbred mice displaying exceedingly wide variations in their degrees of susceptibility to a grafted tumor. Either explanation suggests that nongenetic influences are of considerable importance in the establishment of early transplant generations from primary hepatomas. It will be recalled that one of the objectives of these investigations was to ascertain whether nongenetic factors were of importance in the propagation of hepatomas.

In view of the inability of grafts from primary hepatomas to grow in all mice, and the long latent period required for the first few transplant generations, it is suggested that at least 10 mice be used for each early transfer. This procedure may have increased the number of propagable tumors procured in the present work.

Another biologic property displayed by most of the transplanted hepatomas was their ability to grow faster after the first few animal passages. This is a recognized characteristic of most transplanted tumors and has been noted by all investigators who have carried liver tumors through a series of transplant generations. The present results confirmed this observation and, in addition, showed that the growth rates of the established hepatomas varied considerably. These variations in the growth rates of tumors of similar origin presents an opportunity to ascertain whether they also possess different chemical properties. Such studies

could include the use of both induced and spontaneous hepatomas from the same inbred strain.

An analysis of the properties of the transplantable hepatomas obtained in these experiments revealed that some tumors had a short latent period in first-generation hosts, grew in virtually all inoculated animals and established a rapid growth rate, while others had a long latent period in first-generation hosts, grew in fewer animals and acquired a slower growth rate. These findings suggest that the primary hepatomas may have differed in their degrees of malignancy and that during animal passage most hepatomas acquired greater growth power.

One interesting point in this respect was the inability of 8 hepatomas induced with carbon tetrachloride in strain I mice to grow in any first-generation host. Edwards and Dalton (*10*) transplanted 8 similarly induced hepatomas of strain A mice and obtained success with one only. Eschenbrenner (*27*) of the National Cancer Institute has transplanted 3 hepatomas induced in strain A mice with chloroform and obtained 1 propagable tumor.[6] The 2 hepatomas that failed to grow were transplanted into 10 mice that survived for 12 to 13 months following inoculation. The other was implanted into 24 mice and grew in 2. This tumor was then carried through 54 transplant generations. During the first 18 passages, 429 mice were inoculated of which 173, or 40 percent developed tumors. Perhaps the hepatomas induced by carbon tetrachloride or chloroform are essentially more benign than those induced by *o*-aminoazotoluene. It is known that carbon tetrachloride and chloroform evoke hepatomas only, whereas *o*-aminoazotoluene produces a variety of tumors (*20*).

Since successful transplantation of tumor tissue is not the sole criterion for malignancy in inbred animals, an attempt was made to correlate the biologic activity of the hepatomas with their histologic structure.

Histologic study of all the hepatomas in these experiments, both those that grew and those that failed to grow, was unsuccessful in demonstrating any consistent difference in the two groups. Likewise, a study of the transplant generations revealed no change in morphology which might accompany the increased growth rate, although mitotic figures were frequent in some rapidly growing transplant lines, and infrequent in more slowly growing ones. Such a failure was not unexpected, since Bashford in 1911 (*28*) had made a similar study of morphology and biologic behavior in mammary tumors and concluded that malignant growth was not necessarily associated with loss of differentiation and that retention of what was practically complete differentiation was compatible with malignancy, and was no sure indication of benignancy. He also stated that the morphologic characters of tumor cells were only "correlated characteristics" of cells capable of continuous growth. Hepatomas of the liver have a remarkable similarity histologically to normal liver tissue and this, together with the frequent failure to transplant, is probably the

[6] Unpublished data kindly supplied by Dr. A. B. Eschenbrenner.

basis on which they have often been designated as benign. The morphology of these tumors, however, does show considerable variation over small areas. Cell size, degree of vacuolation of the cytoplasm, content of periodic-Schiff-positive substance, presence of degenerative changes, and degree of fibrosis and vascularity may vary greatly in the same tumor. It is possible that growth potential may vary in the same way, and that an unidentifiable factor of this type may bring success or failure when a series of transfers is made from the same tumor. Since reports of metastasis of hepatomas are rare and since they grow slowly, they may have a limited power for establishing themselves in a new host, and unless all conditions are favorable, transplantation will not succeed, even in mice of the same genetic constitution. The measurement of the size of the tumor generally relied upon in estimating growth rate did not always represent the actual bulk of tumor tissue since the presence of cystic spaces and vascular dilatation of the sinusoids in these tumors increased the measured diameter without representing actual increase of tumor tissue. The long latent period before any palpable tumor was detected in the first-generation transplant is especially hard to explain. This period accounted for much of the time involved in the long period for tumor growth in the first generation. How tumor cells survive during this latent period is as much of a riddle as the situation in clinical medicine, where recurrences or metastases are found many years after removal of a primary tumor. Studies during the latent period of slowly developing tumors like these hepatomas might be helpful in solving this problem.

A remarkable feature of these tumors was the development at the site of implantation of tumors of a different morphology. Six of the 23 hepatomas induced by *o*-aminoazotoluene eventually appeared as hemangioendothelioma in this laboratory. All 6 were in strain C mice, nearly half of the 14 strain C tumors in the experiment. Three (Nos. 7, 8, and 18) of these alterations occurred in the first generation, but others (Nos. 3, 4, and 10) were delayed to the second, fifth, and seventh generations, respectively. One line (No. 10) which changed in this laboratory, persisted as hepatoma in 2 other laboratories. A significant observation in one section from No. 18 was development of hemangioendothelioma at the margin of the hepatoma, and a section from the margin of another hepatoma (No. 10) showed hyperplasia of vascular endothelial cells in the vicinity of the transplanted hepatoma. Although transformation did not occur in this generation of No. 10, this suggests that the presence of the hepatoma stimulated a vascular growth which may have been more frequent than actually observed. No change in the morphology of the hepatoma itself was found preceding the development of hemangioendothelioma and "typical" hepatoma tissue was found in several altered tumors. When changes to sarcoma have been observed in mammary tumors and lung tumors, there is frequently a stage of mixed tumor, or carcinosarcoma preceding the complete alteration. The observation of a change in the contiguous tissue suggests that when hemangioendo-

thelioma developed, the hepatoma induced a change in adjacent vascular tissue leading eventually to neoplasia of this tissue, and persistence of this neoplasm rather than the hepatoma on transplantation caused an apparent change in tumor morphology.

Four of the 13 lines of hepatoma developing spontaneously in the C3H mice also showed development of tumors of different morphology. These were of four different types which were unlike the alterations in strain C mice in which all the newly developed tumors were hemangioendotheliomas. One of them (No. 31) showed a gradual change through several generations with final development of a reticulum-cell sarcoma. Normal reticulum cells within the tumor may have been the site of alteration.

Other transplanted hepatoma lines, however, were carried through many generations and no alteration into tumors of different cell origin was found. This is in agreement with accounts of other "transforming" types of tumors such as mammary tumors and lung tumors, in which some lines have been carried for very long periods, without showing any change in their epithelial character. The fact that separated lines from No. 10 behaved differently, suggests that extrinsic and chance factors may be responsible.

Bashford in discussing the change to sarcomas in mammary tumors posed several questions: "(a) Are these sarcomas true new growths, or infective granuloma? (b) Are they true sarcoma or modified carcinoma cells? (c) Is there a primarily existing mixed tumor? (d) Are they spontaneous sarcomas which accidentally occur in this area?" The question of infective granuloma can be dismissed in considering the change in the hepatomas since a variety of changes developed, none of which resembled granuloma. In answer to the second question it seems highly improbable that the morphology of a hepatoma could be modified to resemble a hemangioendothelioma, an osteogenic sarcoma, a reticulum-cell sarcoma and the two epithelial tumors which occurred in the strain C3H mice. It is true that the primary of one of these tumors (hepatoma 30) showed proliferation of adjacent bile ducts which was unusual, and the change in morphology occurred in the first generation. This may have been, therefore, a cholangioma, which has never been recognized in the mouse. In regard to the third question the possibility of a primary mixed tumor must be seriously considered, especially since 1) the hemangioendotheliomas occurred in strain C mice in which such tumors are readily induced by *o*-aminoazotoluene (*21*), and 2) two of the hepatomas (Nos. 7, 8) which transformed to hemangioendothelioma originated in the same mouse and the change in each occurred during the first generation. On the other hand, the change occurred in three of the transplanted strains at the second, fifth, and seventh generation, and some of these tumors carried in other laboratories behaved differently. The changes in the C3H mice likewise occurred in generations later than the first, and it seems improbable that tumors of such varied

morphology could have existed primarily in the liver. The question of a spontaneous tumor is harder to rule out, but most of the animals in which the new tumors were found were below the age when such tumors are expected. Moreover, these growths arose at the site of the hepatoma transplants, and the animals were free of other tumors. The actual incorporation of hepatoma cells in one of the tumors (No. 32) is especially significant.

It seems that the possibility must be entertained that the slowly growing hepatoma can induce other forms of tumor in a susceptible host. Such a possibility has been offered to explain the transformation of other epithelial tumors and the neoplastic epithelial cell may induce a change in the normal fibroblastic tissue of the stroma (*29*). In two instances the development of the different tumor type was seen to be contiguous to the hepatoma tissue, and no internal change was found in the hepatoma proper preceding the development of the hemangioendothelioma. The fact that the types of tumor newly developing varied with the strain of the host mouse is also compatible with the action of a carcinogenic agent. The "osteogenic" tumor in the C3H mouse in a subcutaneous location is hard to explain, but we have seen such tumors at sites removed from bone. The gradual development of the reticulum-cell sarcoma, and the inclusion of hepatoma tissue in the early stages of the transformation indicates a direct effect on contiguous cells. The two epithelial tumors in the strain C3H could be variants of mammary tumors, possibly of duct origin, since mammary tumors display a diversity of form, but morphologically they were unlike the usual mammary tumors in the mouse.

SUMMARY AND CONCLUSIONS

Eighty-one induced or spontaneous hepatomas arising in inbred mice were transplanted subcutaneously into from 1 to 4 members of their strains of origin: Of 36 hepatomas which grew in first-generation hosts, 31 were propagated through 2 to 15 transplant generations and then discontinued, while 5 failed to grow after 1 to 6 transplant generations.

Most of the successfully propagated hepatomas failed to grow in all recipients. This, together with the inability of 45 primary hepatomas to become established in new hosts and the inability of 5 hepatomas to undergo serial passages, indicates that nongenetic factors were of considerable importance in the transplantability of these tumors.

Of 51 primary hepatomas induced with *o*-aminoazotoluene, 23 arose in strain C mice and 14 grew in new hosts, 4 arose in strain C3H mice and 1 grew, 24 arose in strain I mice and 8 grew.

None of 3 primary hepatomas induced with 3:4:5:6-dibenzcarbazole and 8 induced with carbon tetrachloride in mice of strain I grew in new hosts. These findings with strain I mice suggest that hepatomas

induced with carbon tetrachloride may be less malignant than those induced with *o*-aminoazotoluene.

Of 19 primary spontaneous hepatomas from 15-month-old strain C3H males 13 became established in new hosts.

These findings establish the feasibility of procuring transplantable hepatomas from induced or spontaneous growths.

Most of the successfully propagated hepatomas developed increased growth rates during serial passages, but the growth rates of the established hepatomas varied considerably.

No identifiable qualitative difference could be found on histologic study between spontaneous tumors and those induced by carbon tetrachloride or *o*-aminoazotoluene. An impression was gained that the carbon tetrachloride tumors were less altered from the normal that were the spontaneous tumors, which in turn were less altered than those induced by *o*-aminoazotoluene. This can be proved, however, only by having a large group of these variously derived tumors obtained from the same strains of mice.

Histologic study of the primary hepatomas and all transplant generations did not reveal any consistent difference between those that grew and those that failed to grow in new hosts. Futhermore, the transplant generations revealed no change accompanying the increased growth rates during serial passages.

Six hepatomas induced with *o*-aminoazotoluene in strain C mice either were transformed to or induced hemangioendotheliomas while undergoing transplantation. Four spontaneous hepatomas from strain C3H mice were transformed to tumors of 4 different types, or induced these tumors from the normal cells.

Careful study of these transformations suggested that the slowly growing hepatomas may induce other forms of tumors in susceptible hosts.

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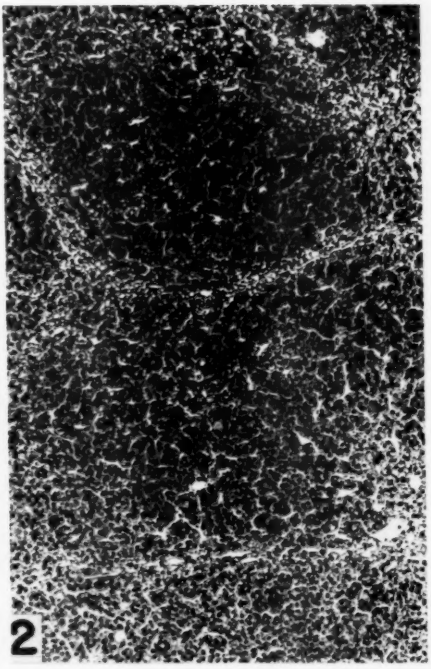
PLATE 48

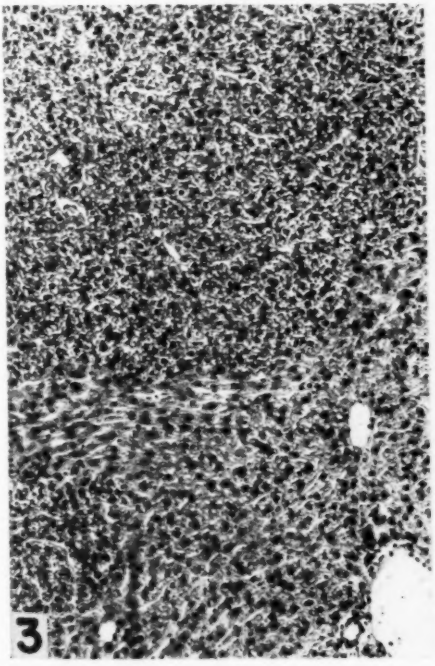
FIGURE 1.—*Hepatoma 6, induced by o-aminoazotoluene, original. Strain I female.* Junction between hepatoma on right and normal liver. Note cystic areas lined by cuboidal epithelium in normal liver adjacent to hepatoma. Large vascular space in upper field. Hematoxylin and eosin. × 100

FIGURE 2.—*Spontaneous hepatoma 29, fifth generation, 43 days growth. Strain C3H male.* Pseudolobulation produced by bands of vacuolated tumor cells and dilated sinusoids. Hematoxylin and eosin. × 100

FIGURE 3.—*Spontaneous hepatoma 27, original. Strain C3H male.* Hepatoma above, composed of cells smaller than normal with more basophilic cytoplasm. A second small nodule of hepatoma tissue lies below and to the left, and the normal liver cells are compressed at the margin. Hematoxylin and eosin. × 100

FIGURE 4.—*Hepatoma 15, induced by o-aminoazotoluene, second generation, 143 days growth. Strain C female.* A peripheral area of hepatoma tissue is composed of small cells, surrounding an area of larger neoplastic cells which are clearly demarcated, producing a "tumor within tumor." Hematoxylin and eosin. × 100





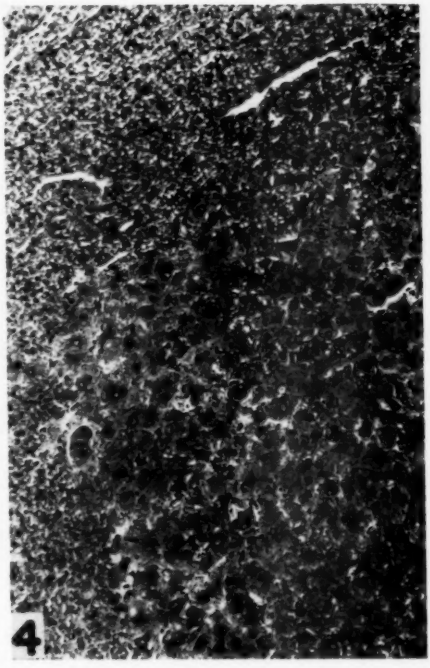


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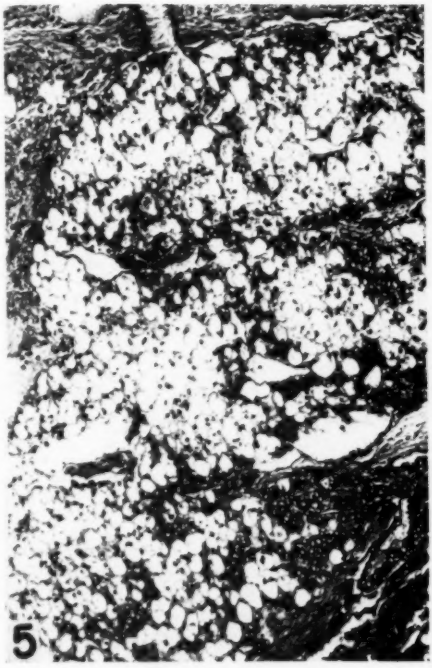
PLATE 49

FIGURE 5.—*Spontaneous hepatoma 27, sixth generation, 36 days growth. Strain C3H male.* Hepatoma showing areas of extremely vacuolated cells resembling adipose tissue. Hematoxylin and eosin. × 100

FIGURE 6.—*Hepatoma 13, induced by o-aminoazotoluene, fifth generation, 40 days growth. Strain C male.* Hepatoma cells showing distribution of basophilic substance in the cytoplasm. Note ringlike arrangement with nucleus at periphery. × 700

FIGURE 7.—*Hepatoma 10, induced by o-aminoazotoluene, third generation, 66 days growth. Strain C female.* Cystic areas filled with finely granular material. Hematoxylin and eosin. × 100

FIGURE 8.—*Hepatoma 21, induced by o-aminoazotoluene, third generation, 52 days growth. Strain I female.* Areas within a hepatoma, where growth of granulation tissue has filled cystic spaces. Hematoxylin and eosin. × 100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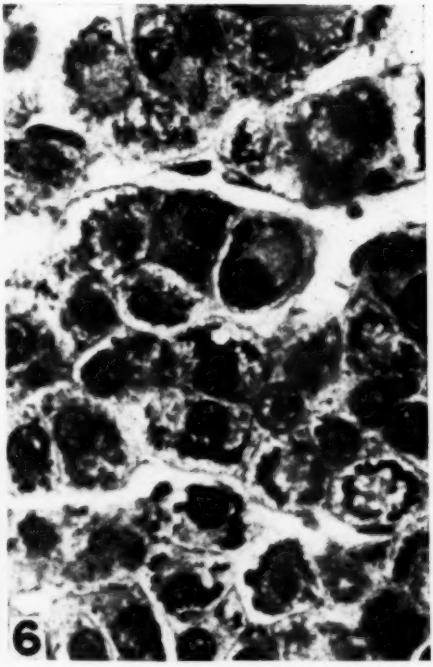
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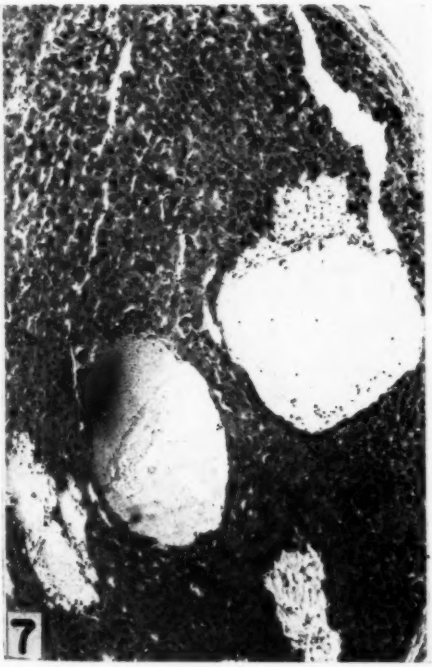
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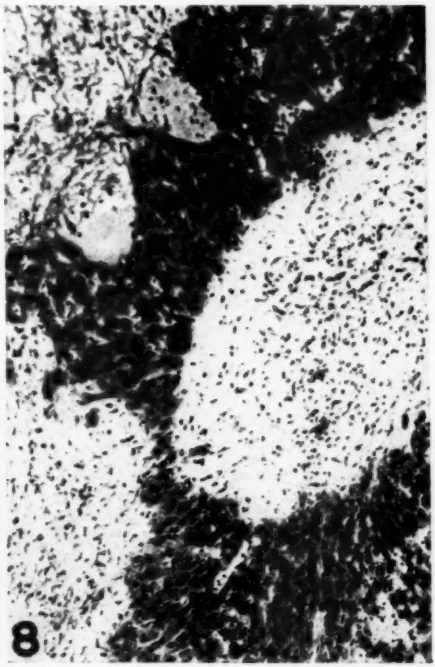
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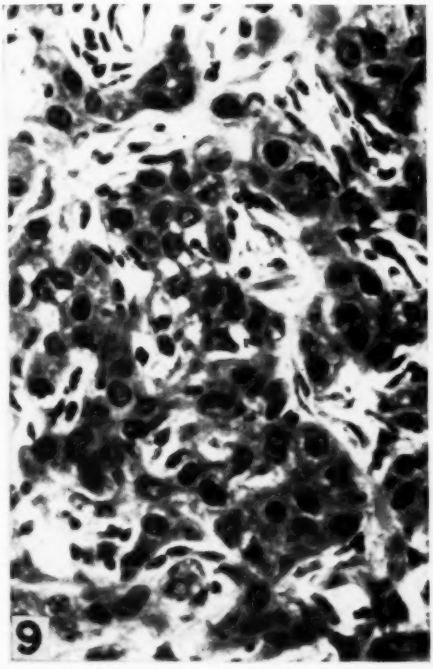
PLATE 50

FIGURE 9.—*Hepatoma 11, induced by o-aminoazotoluene, original. Strain C male.* Proliferation of fusiform cells between cords of hepatoma cells. Hematoxylin and eosin. × 420

FIGURE 10.—*Spontaneous hepatoma, original. Strain I male.* Hyaline cytoplasmic inclusion bodies ("waxy" bodies). Note large cytoplasmic masses, some of which appear "rimmed" and have a central depression, and empty spaces where bodies have dropped out. Space surrounding bodies is probably result of shrinkage. Some cells show numerous small masses, and a granular cytoplasm. Hematoxylin and eosin. × 700

FIGURE 11.—*Hepatoma induced by o-aminoazotoluene, original. Strain C3H male.* Erythropoiesis. Note clump of nucleated red cells in hepatoma tissue on left. No granulocytic cells were found. Hematoxylin and eosin. × 400

FIGURE 12.—*Spontaneous hepatoma 29, second generation, 229 days growth. Strain C3H male.* Note isolated megakaryocytes in hepatoma. Hematoxylin and eosin. × 400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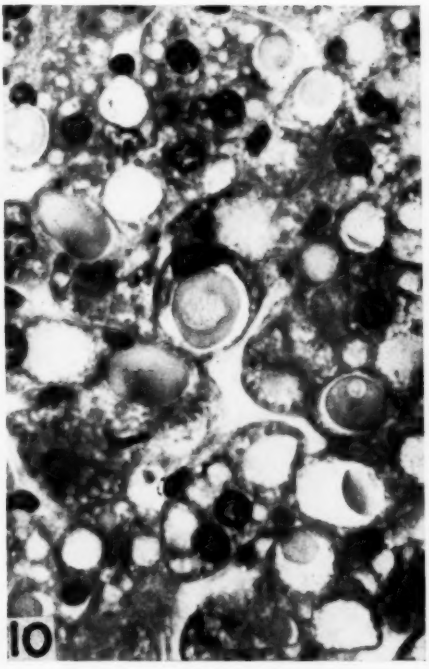
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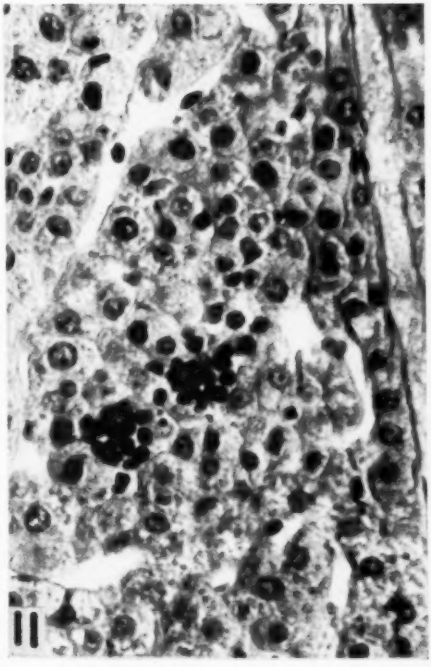
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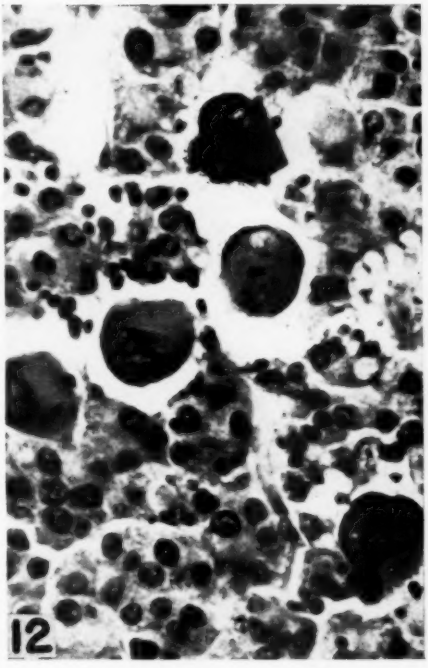
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PLATE 51

All sections Figures 13 to 16 stained by periodic acid-Schiff technique.

FIGURE 13.—*Spontaneous hepatoma 30, original. Strain C3H male.* Increased glycogen in hepatoma on right. Nontumorous liver on left. × 40

FIGURE 14.—*Spontaneous hepatoma 29, original. Strain C3H male.* Tumor cells were of small size. Note increased amount of glycogen in parts of tumor. × 50

FIGURE 15.—*Hepatoma 17, induced by o-aminoazotoluene, original. Strain C male.* Note hepatoma above with large cells which contain relatively less glycogen than nontumorous liver cells below. Hematoxylin and eosin. × 70

FIGURE 16.—*Hepatoma 1, induced by o-aminoazotoluene, eleventh generation, 14 days growth. Strain I female.* Note abrupt change from an area free of glycogen to an area where glycogen was abundant. No morphologic difference in these areas was detected in hematoxylin and eosin staining. × 70

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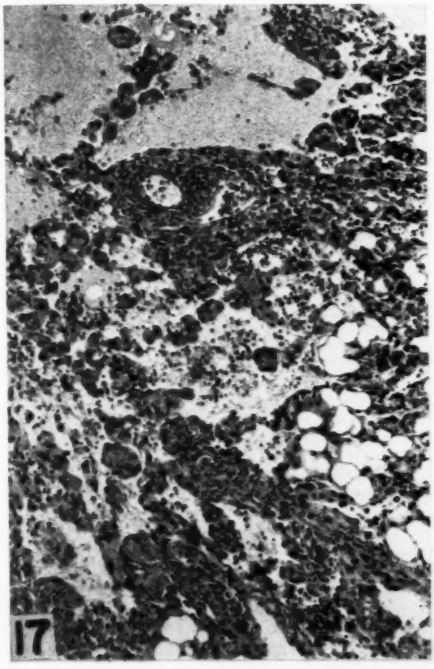
PLATE 52

FIGURE 17.—*Tumor arising from hepatoma 3, second generation, 74 days growth. Strain C female.* Hemangioendothelioma. Large vascular spaces are present, and neoplasm is infiltrating adipose tissue on the left. Hematoxylin and eosin. × 100

FIGURE 18.—*Tumor arising from hepatoma 18, fourth generation, 35 days growth. Strain C female.* Hemangioendothelioma in which a large central blood-filled cyst was formed, surrounded by a narrow wall of hemangioendothelioma cells and an outer capsule of connective tissue. Hematoxylin and eosin. × 100

FIGURE 19.—*Primary hemangioendothelioma arising subcutaneously in mouse which also developed hepatoma 18.* Tumor tissue in upper right, congestion and hemorrhage in neighboring adipose tissue at lower left. × 100

FIGURE 20.—*Tumor arising at margin of hepatoma 10, second generation, 118 days growth. Strain C female.* Characteristic hepatoma tissue at upper left, and contiguous hemangioendothelioma separated by a narrow band of fibrous tissue. Hematoxylin and eosin. × 100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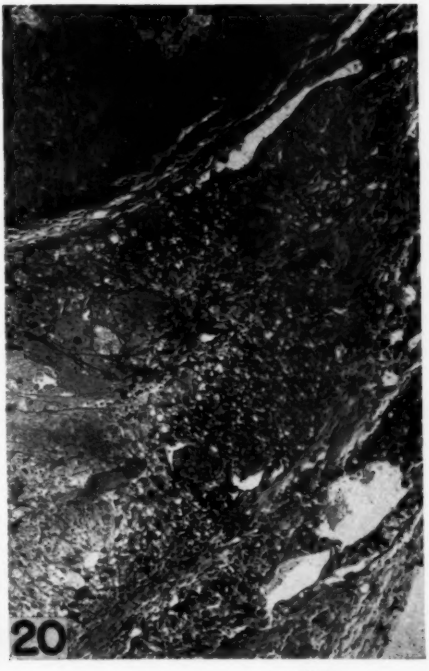
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PLATE 53

FIGURE 21.—*Hepatoma 15, induced by o-aminoazotoluene, original.* Hepatoma area of unusual structure. Note circles of cuboidal cells surrounding large finely vacuolated cells. Hematoxylin and eosin. × 100

FIGURE 22.—*Tumor arising from spontaneous hepatoma 29, sixth generation, 34 days growth. Strain C3H male.* Tumor in which neoplastic cells are arranged in wide cords. The nuclei of the cells lie at the periphery. One focus suggests early ossification. Hematoxylin and eosin. × 100

FIGURE 23.—*Spontaneous hepatoma 30, original. Strain C3H male.* Hepatoma cells at upper right surrounded by a zone of fibrosis, and glandlike areas suggesting bile duct proliferation. Hematoxylin and eosin. × 100

FIGURE 24.—*Spontaneous hepatoma 30, first generation, 341 days growth. Strain C3H male.* Tumor developed at site of transplantation of tumor shown in figure 23. Note neoplastic epithelial cells covering bands of coarse connective tissue. Hematoxylin and eosin. × 100

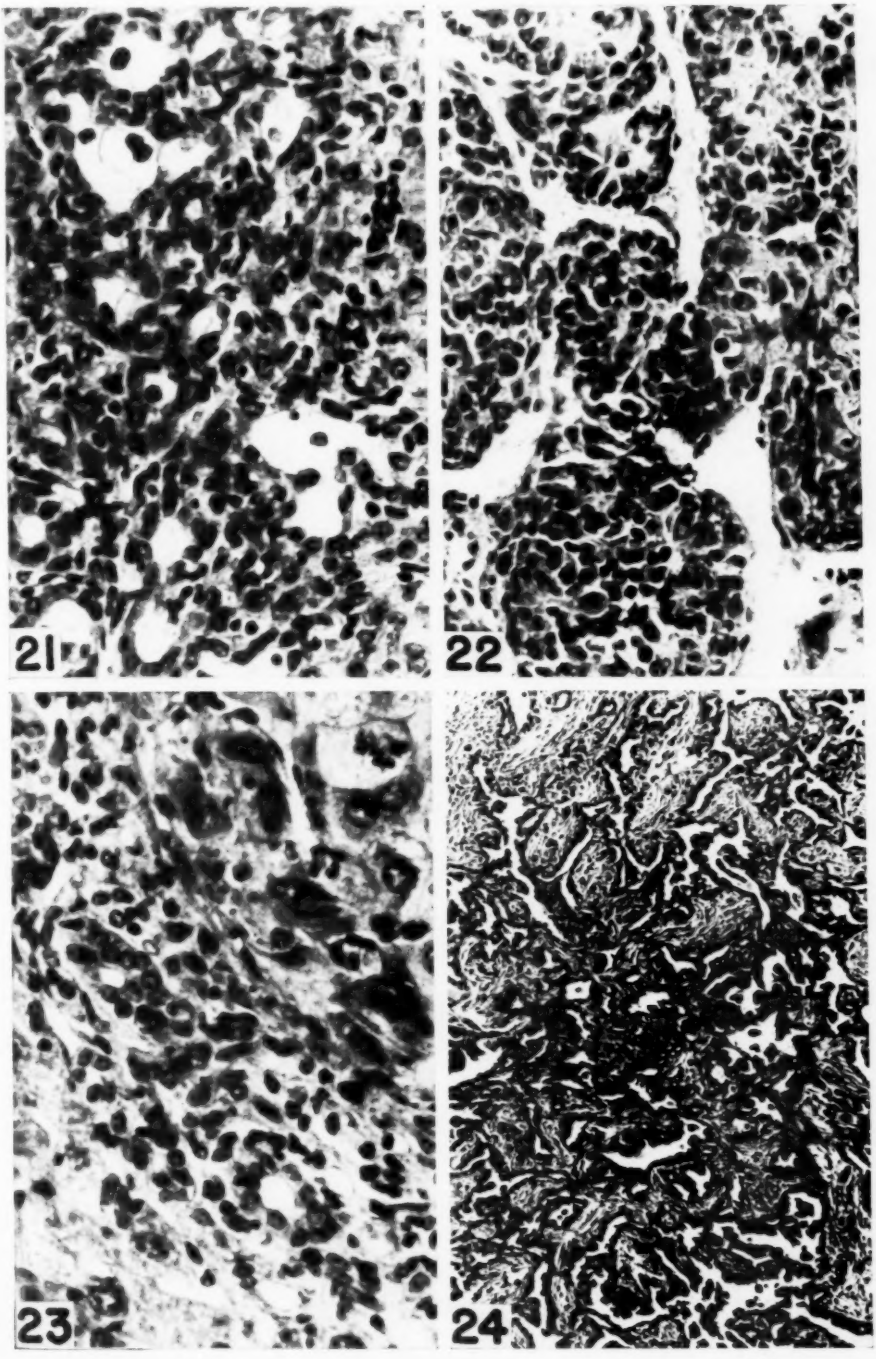
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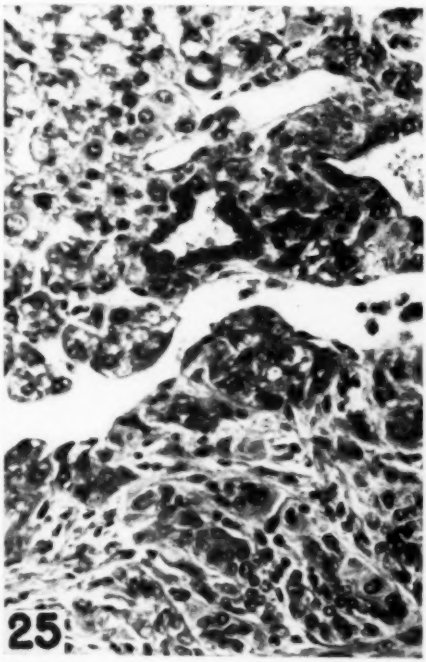
PLATE 54

FIGURE 25.—*Tumor arising from spontaneous hepatoma 31, first generation, 328 days growth. Strain C3H male.* Characteristic hepatoma tissue appears in upper field, some glandular areas lie below this, and tissue composed of large fusiform cells is in lower field. Hematoxylin and eosin. × 300

FIGURE 26.—*Later generation (sixth, 12 days growth) of tumor shown in figure 25.* Tumor tissue composed of fusiform cells encircles a cystic space, the walls of which are composed of rounded cells, many of which are detached, and lie free. Hematoxylin and eosin. × 100

FIGURE 27, A and B.—*Two areas from tumor 31, twenty-second generation of tumor shown in figure 25.* (A) Note rounded cells with conspicuous clear central area in the cytoplasm, and (B) fusiform cells infiltrating muscle. This variable cell formation is characteristic of reticulum-cell sarcomas of the mouse. Hematoxylin and eosin. × 475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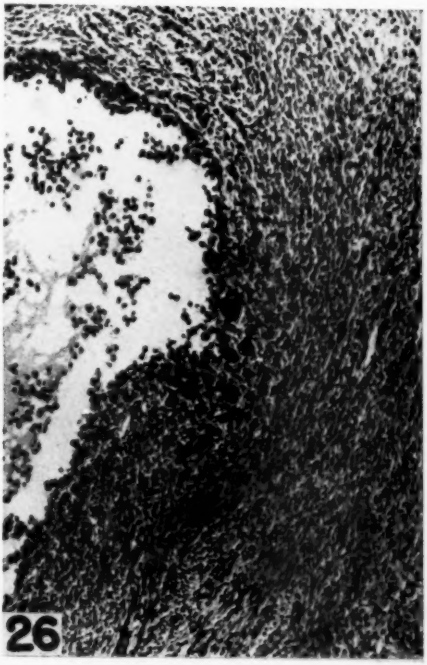
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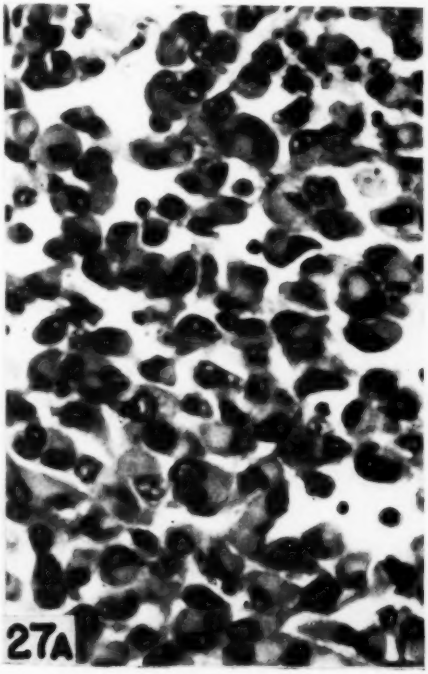
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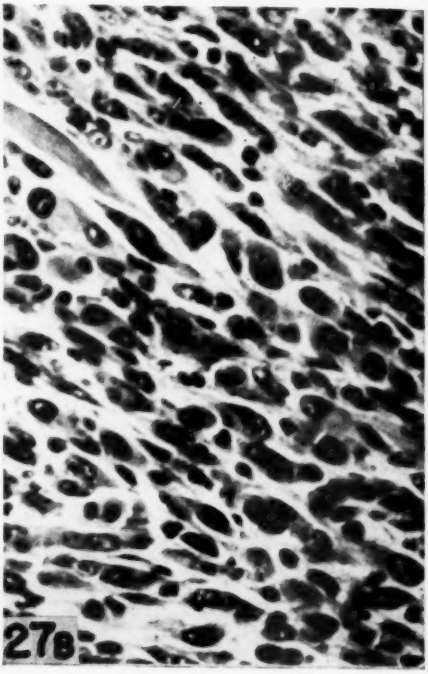
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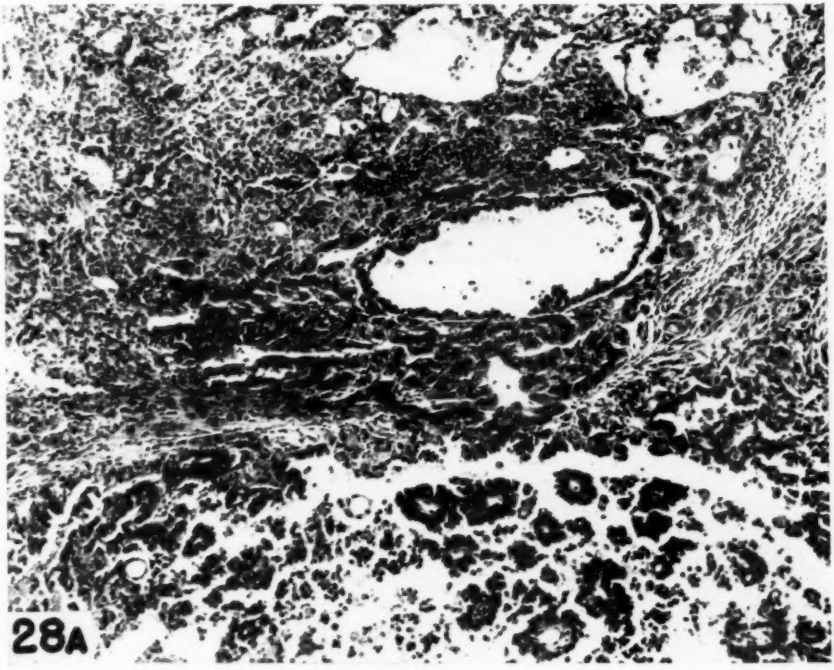
PLATE 55

FIGURE 28, A and B.—*Tumor arising at site of transplantation of spontaneous hepatoma 32, second generation. Strain C3H female.* Epithelial neoplasm of undetermined origin. (A) Lower magnification (× 100) shows general adenocarcinomatous structure. A small area of hepatoma cells appears in the center below an epithelial lined space. (B) Higher magnification (× 475) of hepatoma cells. Note characteristic liverlike cells arranged in cords. Hematoxylin and eosin.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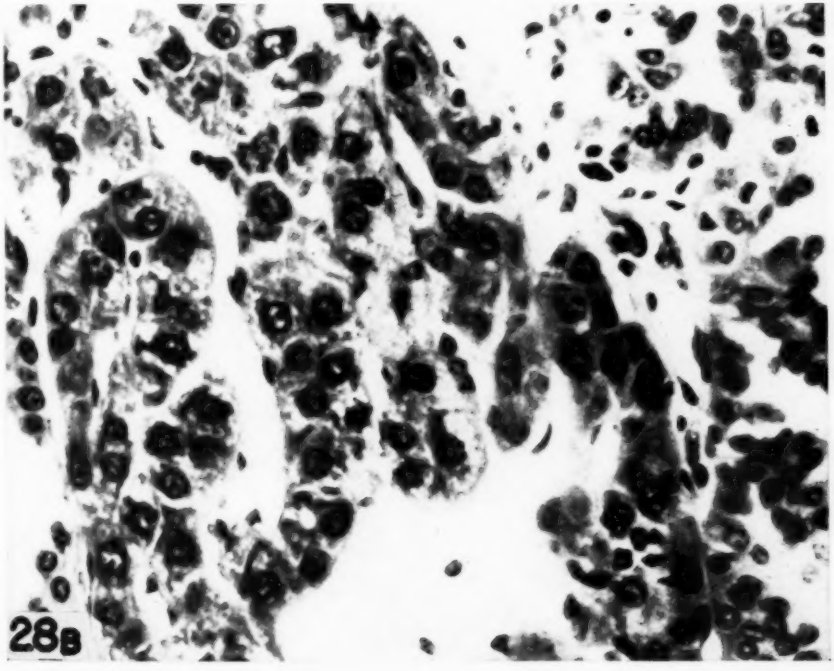
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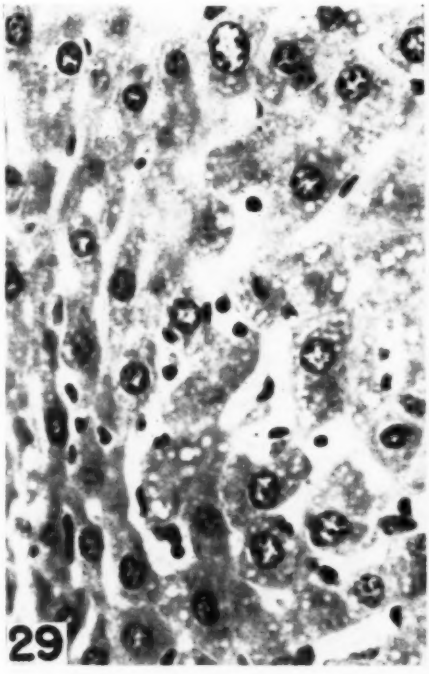
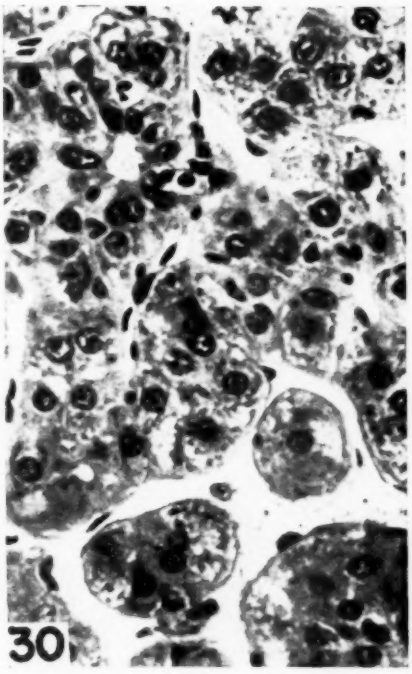
PLATE 56

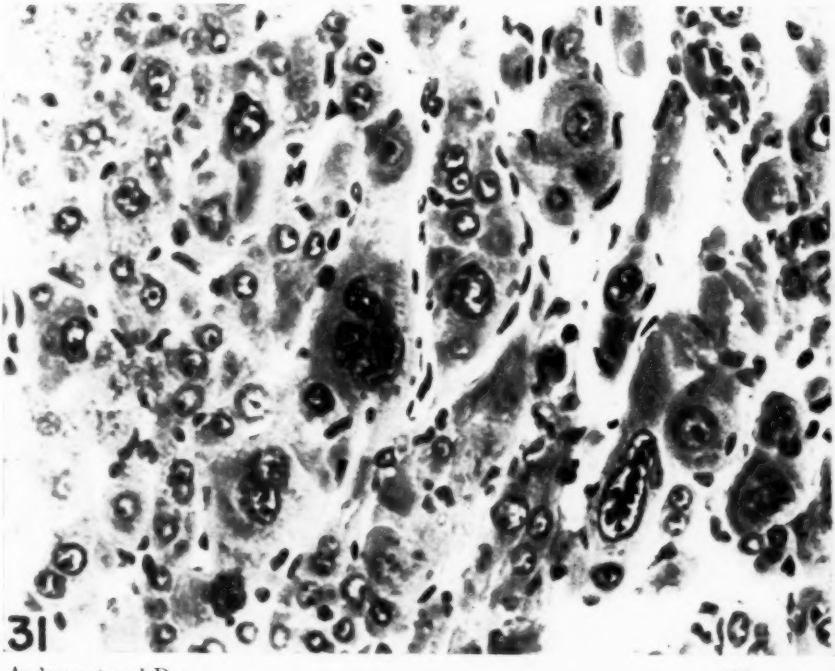
FIGURE 29.—*Hepatoma induced by carbon tetrachloride, original. Strain I female.* Hepatoma cells on right show close similarity to non-neoplastic hepatic cells, which appear in lower left. No clear demarcation is observed. Hematoxylin and eosin. × 420

FIGURE 30.—*Spontaneous hepatoma 28, original. Strain C3H male.* Hepatoma area from center of a large tumor, showing considerable alteration from normal liver tissue. Cell cytoplasm is increased, although nuclear size appears normal. Hematoxylin and eosin. × 420

FIGURE 31.—*Hepatoma 16, induced by o-aminoazotoluene, original. Strain C female.* Some hepatoma cells are of giant size and have multilobed nuclei. This represents a marked alteration from nontumorous liver. Hematoxylin and eosin. × 420







216903—52——28

Studies on the Development of Mammary Tumors in Dilute-Brown DBAb Mice Without the Agent [1]

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Since Bittner's discovery (*1*) that infection with the mammary tumor agent takes place via the mother's milk, mice of high-mammary-tumor strains have been nursed in many laboratories by foster mothers belonging to low-mammary-tumor strains. These foster-nursed animals showed a low tumor rate. The generally used method of ensuring that the newborn do not drink milk from the mother consists in operative removal of the young from the uterus immediately before birth, whereupon they are placed with a low-cancer-strain foster mother for nursing. In this manner, lines without the mammary tumor agent were developed from the cancer strains most frequently used. Bittner (*2*) found no mammary tumors in the agent-free A and C3H strains of his colony. We have recently reported (*3*) that the breeding females of our line of the agent-free A strain were also free of mammary tumors; 66 females died without mammary tumors at an average age of 607 days. Heston (*4*, *5*) investigated the agent-free C3Hb strain, among which a relatively high frequency of mammary tumors was observed. Andervont and Dunn (*6*, *7*) report on the incidence of mammary tumors in an agent-free line of the DBA strain. In 7 completed generations, comprising 174 breeding females that lived to an average age of 20 months, only one developed a mammary tumor. This paper describes the mammary tumor incidence in our agent-free DBAb line in the first 7 completed generations; various factors of importance in the development of these tumors are analyzed and the morphologic aspects described.

Experiments on the Transmission of the Agent In Utero

Transmission of the mammary tumor agent in high-cancer strains takes place via the mother's milk. Young removed from the uterus of pregnant

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females shortly before birth, and nursed by foster mothers belonging to an agent-free strain, may therefore be expected to possess no mammary tumor agent, although the natural mother did. Lines of high-cancer strains without the agent were therefore started with the aid of this method. It is essential, however, to exclude the possibility of intra-uterine transmission of the agent from the mother to the young. A second mode of infection should be taken into account. The mammary tumor agent was demonstrated in the sperm (*8*) and the seminal vesicles (*6*) of high-cancer-strain mice. Intra-uterine infection of the embryo with the agent from the sperm is therefore by no means impossible. A recently published investigation (*9*) failed to reveal evidence of this mode of infection in the DBA strain and its hybrids; however, this possibility is not excluded with certainty. The results of numerous experiments, in various laboratories, with elimination of transmission via the milk are suggestive of inability of the mammary tumor agent to cross the placental barrier. In all cases, elimination of the milk influence resulted in a marked decrease in the mammary tumor rate.

However, the results obtained by Fekete and Little (*10*) with reciprocal transference of fertilized ova between female mice of high- and low-cancer strains suggest that intra-uterine transmission of the agent is possible. Dmochowski (*11*) examined the problem from a different angle. C3H high-breast-cancer strain embryos and placentae were tested for the presence of the mammary tumor agent. While embryos of these high-cancer females did not show the presence of the agent, placentae of these mice contained the milk factor only in very small amounts. Our DBA strain was submitted to the same tests. In order to ascertain that the donor animals did possess the tumor agent, only females with mammary tumors were used for the tests. At the end of pregnancy (on the 18th to 19th day), the young were removed from the uterus of 4 different donor animals. After careful cleansing, the organs of the fetus were taken from the thoracic and abdominal cavities and ground with sand; 5 ml. of distilled water was added to each gram of fresh tissue. The mixture was spun for 10 minutes in an ordinary laboratory centrifuge. Test animals 3 to 4 weeks old were immediately given an intraperitoneal injection of 0.1 ml. of the supernatant fluid. After rinsing in water, a placental extract was prepared in the same manner, and 0.1 ml. of tumor extract (1 gram of tissue in 5 ml. of water) from the same animal was tested for comparison. Other test animals received immediate intraperitoneal injection of 0.1 ml. of blood from the carotid artery. Other test animals were submitted to subcutaneous implantation of a piece of spleen. Tumor extracts, blood, and spleen from nonpregnant Dilute-brown females were tested in the same manner for comparison. The test animals used were F_1 hybrids obtained by mating O_{20} females with agent-free DBAb males

(Odz). The females were force-bred with their brothers for one year and then watched for the development of mammary tumors. Odz males were castrated after weaning and given estrone pellets (25% estrone and 75% cholesterol) subcutaneously. The results are given in table 1. The results show that the mammary tumor agent is not demonstrable in the embryos. The placental extract revealed mammary tumors in about 50 percent of the test animals. The tumor agent was found in all tumor extracts, and in the blood and the spleen of both the nonpregnant animals, and the pregnant animals at the end of term. These findings substantiate the theory that the mammary tumor agent is not transmitted from the mother to the embryo *in utero* and that intra-uterine "infection" by the father's sperm does not take place. It should be borne in mind, however, that these negative results do not constitute absolute proof of the absence of the agent. The agent may be present in quantities too small to be demonstrated by the method used.

TABLE 1.—*Mammary tumor agent in nonpregnant and pregnant DBA females with mammary tumors*

Donors	Test animals	Total number of mice	Mice bearing mammary tumors		Mice without mammary tumors	
			Number	Average age with tumor (days)	Number	Average age at death (days)
♀ *DBA, nonpregnant*						
Mammary tumor extract	♀ Odz	30	27	331	3	346
	♂ Odz	9	5	293	4	435
Blood	♀ Odz	7	7	342	....	....
	♂ Odz	5	3	309	2	388
Spleen	♀ Odz	4	3	322	1	566
	♂ Odz	6	3	320	3	325
♀ *DBA, pregnant* à terme						
Tumor extract	♀ Odz	2	2	270	....	....
	♂ Odz	10	8	324	2	360
Blood	♀ Odz	5	5	343	....	....
Spleen	♀ Odz	2	2	323	....	....
	♂ Odz	3	3	304	....	....
Placenta	♀ Odz	8	4	355	4	632
	♂ Odz	10	7	324	3	533
Embryos, *ex utero*	♀ Odz	17	2	493	15	687
	♂ Odz	7	1	341	6	458
Controls, untreated	♀ Odz	122	7	749	115	752
	♂ Odz	24	1	606	23	574

The results of this investigation are not in accordance with those obtained by Hummel and Little (*12*), who found no mammary tumor agent in the blood at the end of pregnancy. This discrepancy awaits explanation by further investigation.

Origin of the DBAb Line

The DBAb line originates from the Dilute-brown strain (DBA) kept at our Institute. Dr. Korteweg received this strain from Dr. Little in 1931. The mammary tumor rate in recent years is shown in table 2.

Table 2.—*Mammary tumor incidence of females of strains DBA, C57BL, and DBAb*

Strain	Total number	Average number of litters	Tumor-bearing		Non-tumor-bearing
			Percent	Average age (*days*)	Average age at death (*days*)
DBA (virgins)	86	.....	59	546	537
DBA (breeders with 3 and more litters)	151	6	68	408	466
DBA (force-bred with 1–2 litters)	48	2	62	477	586
DBA (force-bred with 3 and more litters)	69	7	97	382	533
C57BL (virgins)	193	.....	0	.....	621
C57BL (breeders with 3 and more litters)	70	6	0	.....	589
DBAb (virgins)	96	.....	4	649	650
DBAb (breeders with 1–2 litters)	60	1	1	541	595
DBAb (breeders with 3 and more litters)	120	6	12	564	604
DBAb (force-bred with 1–2 litters)	31	1	16	638	559
DBAb (force-bred with 3 and more litters)	135	7	15	503	581

The DBAb line was started with 3 females and 1 male, which were operatively removed from the uterus of a DBA female immediately before birth. This litter was foster-nursed by a female of the C57BL strain in which no mammary tumors have been observed in recent years (see table 2). Various investigations in recent years failed to demonstrate the mammary tumor agent in the C57BL strain (*8*, *13*).

The three females of the original litter of the DBA female were mated to their brother; in subsequent generations, the line has been maintained by brother-sister matings, exclusively littermates. With the exception of the original litter, the young in the DBAb line were nursed by the natural mothers.

Mammary Tumor Incidence in the DBAb Line

This report concerns 442 females from the first 7 generations of the DBAb line. The animals from the DBAb line listed in table 2 include only those that survived to the age of 12 months. The mice were kept in glass jars and fed commercial pellets and tap water *ad libitum*. Observations were made at weekly intervals, tumors being recorded as appearing on the day when first palpable. Histologic examinations were made in all cases.

Virgin, breeding, and force-bred females were kept. As it is known (*14*) that the number of litters influences the mammary tumor incidence in the DBA strain, the breeding females were divided into two groups: one with 1 or 2 litters, and one with 3 or more. After weaning, the virgin females

were kept 4 in a cage. The breeding females were permanently kept one female with her brother. For force-breeding, 4 females were kept with 1 male; the young were removed as soon as possible after birth. At the end of the breeding period, 4 females were kept in a cage. Autopsy was carried out as soon as a tumor appeared and when the animal became moribund or was found dead.

The mammary tumor incidence of all females is given in table 2. The tumor incidence in virgin females was 4 percent at an average age of 649 days; in breeding females with more than 2 litters, it was 12 percent at an average age of 564 days. This difference is significant. The difference in the tumor age between these two groups is considerable. Only one of 60 breeding females with 1 litter developed a mammary tumor. There is no difference in the mammary tumor percentage between breeding and force-bred females with 3 and more litters. Force-bred females with 1 litter had the same incidence as those with an average of 7 litters. The tumor age in the latter group was considerably earlier.

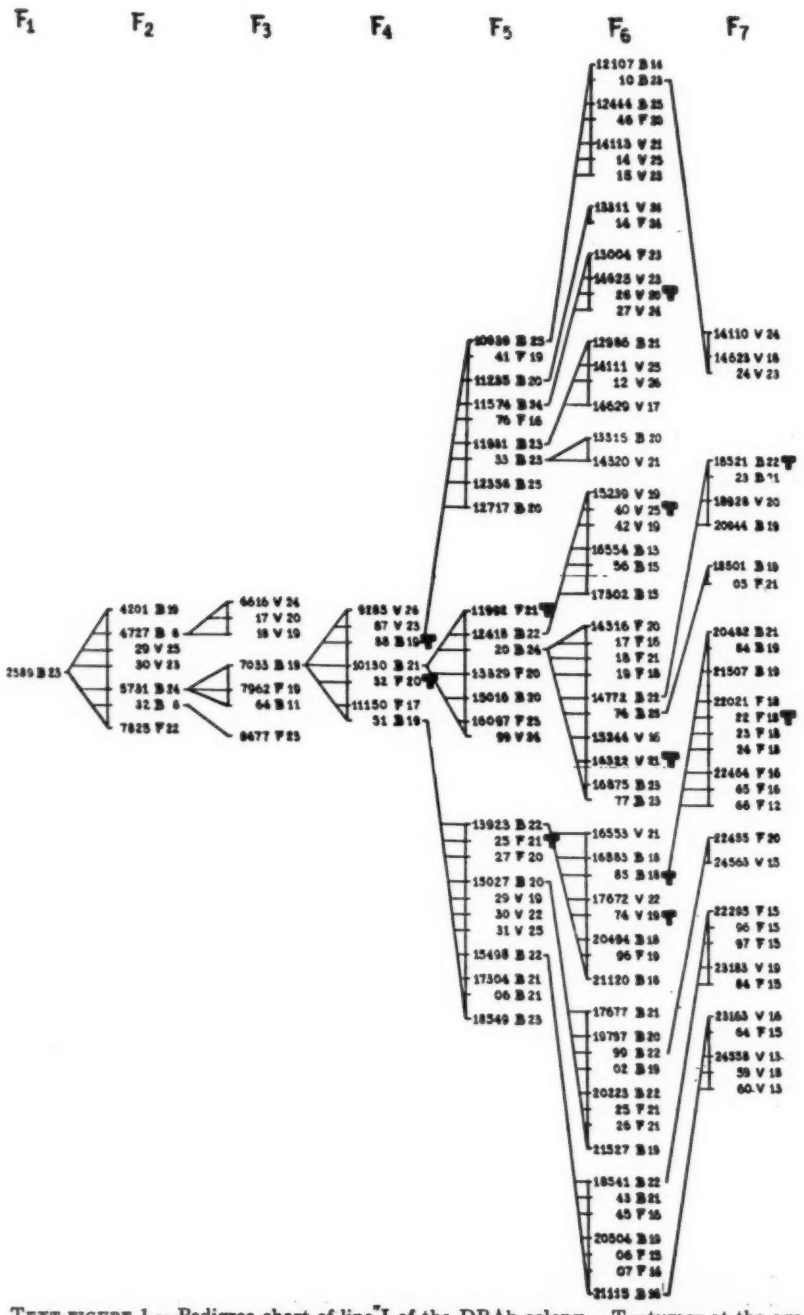
None of the animals of the original litter developed a mammary tumor. The mammary tumor incidence of the 3 separate lines is given in table 3. In order to ensure that the data are comparable, only breeders with more than 2 litters are included in the table. The frequency of mammary tumors shows no differences, with the exception of the breeding females of line II, in which it was slightly higher than in the other lines. No explanation can be given. The average age of the breeders without tumors was the same in all lines.

TABLE 3.—*Number of mammary tumors in DBAb females of lines I, II, III*

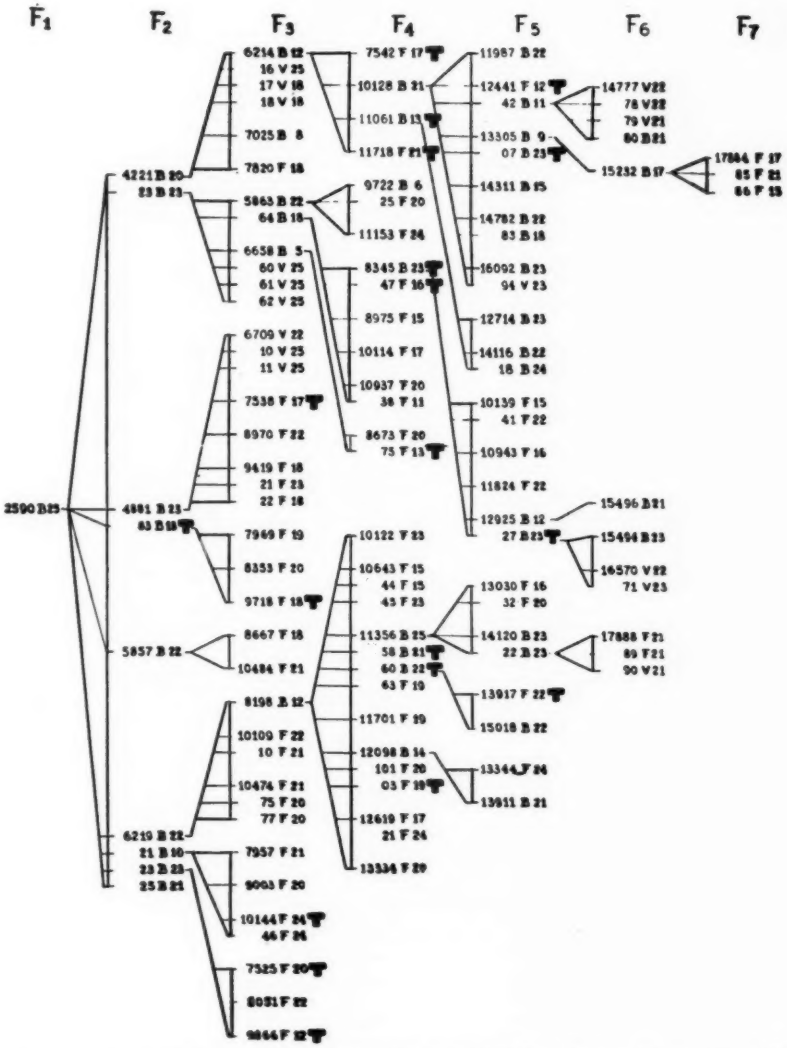
Line	Virgin mice		Breeders with more than 2 litters		Force-bred mice	
	Number of mice	Tumor-bearing	Number	Tumor-bearing	Number	Tumor-bearing
I. No. 2589	21	1	13	1	8	0
II. No. 2590	16	0	31	7	59	12
III. No. 2591	35	1	42	1	54	9

PARENT-OFFSPRING CORRELATION

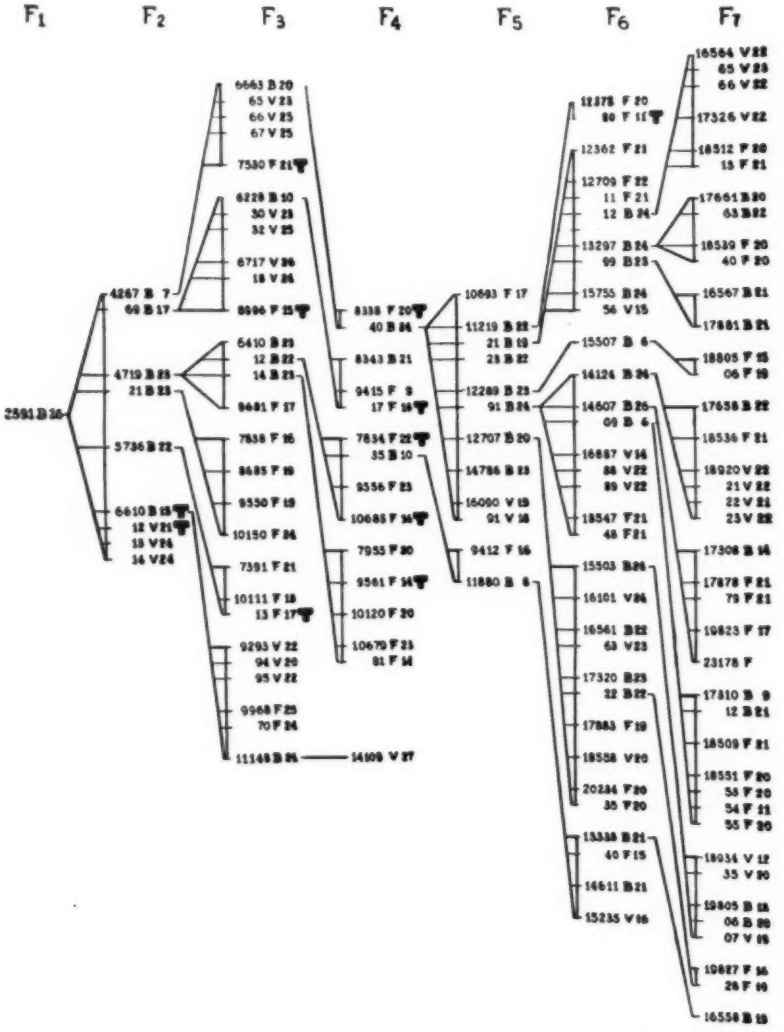
In order to gain some insight into the distribution of mammary tumors and the problem of whether the mammary tumor agent may be responsible for the development of mammary tumors, pedigree charts were made of the female mice of three lines (text-figs. 1–3). These text-figures show that the mammary tumors are distributed at random; excessive numbers of mammary tumors in certain families were not observed. An analysis of the mammary tumor frequency in the daughters of females with and without mammary tumor also fails to reveal any differences between the two groups. Females without tumors had 125 daughters without and 17 with mammary tumors; females with a mammary tumor



TEXT-FIGURE 1.—Pedigree chart of line I of the DBAb colony. T = tumor at the age in months indicated. V = virgins; B = breeders; F = force-bred.



TEXT-FIGURE 2.—Pedigree chart of line II of the DBAb colony. T=tumor at the age in months indicated. V=virgins; B=breeders; F=force-bred.



TEXT-FIGURE 3.—Pedigree chart of line III of the DBAb colony. T = tumor at the age in months indicated. V = virgins; B = breeders; F = force-bred.

had 23 daughters without and 3 with mammary tumors. This computation includes only the daughters kept as breeders or force-breeders. If the agent were of importance in the development of these mammary tumors, the daughters of females with mammary tumors could be expected to show a higher mammary tumor incidence than the daughters of females without mammary tumors.

In a recent paper (*9*) it was emphasized that it is possible that in certain cases with a low amount of tumor agent or with a slow multiplication of the agent an excessive number of mammary tumors may occur in females born in later litters. As can be seen from table 4, there is no such increase in mammary tumors in DBAb breeding females born in later litters. This supports the assumption that the mammary tumor agent is not involved in the development of the mammary tumors in DBAb females.

TABLE 4.—*Mammary tumor incidence in DBAb breeding females according to the number of the litter in which they are born*

	Number of tumor-bearing mice	Percentage of tumors
1st litter	37	13. 8
2nd "	61	3. 3
3rd "	51	19. 6
4th "	42	7. 1
5th "	40	20. 0
6th "	21	19. 0
7th "	23	13. 0
8th "	14	7. 1
9th "	3	0
10th "	9	0
11th "	1	0

TEST FOR THE PRESENCE OF THE MAMMARY TUMOR AGENT IN TUMORS FROM DBAb AND DBA FEMALES

Twelve mammary tumors from DBAb females were tested for the presence of the tumor agent. The tumors were ground with sand and distilled water was added (1 gm. of tumor tissue to 5 ml. of water). After spinning in an ordinary laboratory centrifuge, 0.1 ml. of the supernatant was injected intraperitoneally into sensitive test animals aged 3 to 4 weeks. The test animals were F_1 hybrids obtained by mating O_{20} females to DBAb males (Odz). The females were force-bred with Odz males for one year and then watched for the appearance of mammary tumors. Odz males were castrated after weaning and injected subcutaneously with estrone pellets (25% estrone and 75% cholesterol). Mammary tumors from DBA females were examined in a similar manner, as were also two mammary tumors from DBAb females, which at the age of 4 weeks had been given the similarly prepared agent of a tumor from a DBA female. The results are given in table 5. The untreated control animals were the same as those listed in table 1.

TABLE 5.—*Test for the presence of the mammary tumor agent in mammary tumors from DBAb and DBA female mice.*

Case No. of female donor with mammary tumor	Age (days)	Total number of mice	Test animals: ♀ Od₂ and ♂ Od₂			
			Tumor-bearing		Non-tumor-bearing	
			Number of mice	Average tumor age (days)	Number of mice	Average age at death (days)
DBAb females (without agent)						
4883	560	6 ♀	1	540	5	636
		2 ♂	0	.....	2	429
6610	425	12 ♀	0	.....	12	619
7525	624	2 ♀	0	.....	2	521
		2 ♂	0	.....	2	395
7530	665	3 ♀	0	.....	3	657
		2 ♂	0	.....	2	561
9718	546	3 ♀	0	.....	3	440
		1 ♂	0	.....	1	421
9966	377	1 ♀	0	.....	1	624
		2 ♂	0	.....	2	457
10113	537	3 ♀	2	546	1	618
		3 ♂	0	.....	3	471
10132	598	4 ♀	1	570	3	583
		4 ♂	0	.....	4	476
10685	486	1 ♀	0	.....	1	700
		2 ♂	0	.....	2	508
11061	406	3 ♀	0	.....	3	611
		2 ♂	0	.....	2	568
12380	352	2 ♀	0	.....	2	499
		1 ♂	0	.....	1	293
12441	378	3 ♀	1	385	2	602
		1 ♂	0	.....	1	396
DBA females (with agent)						
419	478	3 ♀	3	473	.....	.....
1390	553					
1794	390	2 ♀	2	432	.....	.....
5399	425					
4972	349	2 ♀	1	300	1	328
3975	442	2 ♀	1	414	1	465
6498	299					
7626	498	4 ♀	4	299	.....	.....
		3 ♂	2	278	1	510
9391	393	8 ♀	8	303	.....	.....
		3 ♂	2	316	1	289
7135	439	8 ♀	8	331	.....	.....
8093	469	2 ♂	1	304	1	669
9766	396					
DBAb females (injected with agent)						
11247	423	4 ♀	3	397	1	544
		1 ♂	1	288	.....	.....
11248	421	2 ♀	2	411	.....	.....
		1 ♂	1	340	.....	.....

Mammary tumors were found in only 5 of 65 test animals injected with an extract of mammary tumors from DBAb females. This percentage of tumors corresponds with that found in untreated control animals (see table 1). In contrast to this, mammary tumors developed in 39 of 45 test animals treated with tumor extract from DBA females or DBAb females injected with the agent. The results therefore show that the mammary tumors from untreated DBAb females do not, unlike the tumors from DBA females, possess the mammary tumor agent.

Morphologic Aspects of Mammary Carcinomas

The morphologic aspects of mammary tumors in the DBAb line were studied and compared with those of tumors occurring in the DBA strain in the presence of the agent. The morphology of mammary tumors in mice with the agent has been known since Apolant's and Murray's publications; it was recently described in summary by Cloudman (*15*) and Dunn (*16*). The microscopic aspects of these tumors are highly variable, and transitional forms occur. The majority of these tumors show one or more of the following 3 structural characteristics: *a*) the tumor cells form acini; *b*) the tumor cells grow in the form of solid trunks and cellular masses; and *c*) cysts lined with tumor cells develop in the tumor tissue.

These forms may show secondary characteristics such as degenerative cellular change, necrosis and cavernous distension of the blood and lymph vessels. In our DBA line, the acinar structure is most markedly exhibited in older animals (15 or more months of age). In younger animals, solid cellular proliferation masses generally predominate. Tumors with a structure different from the above-mentioned acinar structure and its varieties are relatively rare. The following deviating forms were observed: *a*) Tumors with an abundance of keratinized squamous epithelium. Localized epithelial keratinization was not infrequently found in these tumors, the remaining part of which showed an ordinary acinar or related structure. Occasionally, however, tumors were observed in which squamous metaplasia was so pronounced that the picture was predominated by squamous epithelium and keratinized masses, while local patches of adenocarcinoma, solid carcinoma, or acinar carcinoma were found only after careful examination. *b*) Pronounced papillary proliferation in adenocystic tumors. *c*) Adenocarcinomas with tubular formations and clefts, as a rule with a relatively large quantity of cellular connective tissue between the epithelial formations. *d*) Among the tumors found in DBA mice, there were a few that were slightly different from the majority of acinar and related tumors. A few solid tumors were found, for instance, which consisted of cells larger than those of the other tumors; a few other tumors showed dissolution of the tumor cells; there were also tumors with an abnormally cellular and irregular stroma, and a few adenocarcinomatous forms with an unusual type of cell. In some of these forms, we considered it to be dubious whether the deviations from the usual varieties

of acinar carcinoma depend upon secondary factors such as degenerative changes as a result of a deficient blood supply, etc., or actually represent an expression of deviating structural potencies of the carcinoma cells.

In our DBA strain, about 10 percent of the total number of tumors deviated from carcinomas with an acinar structure and their solid and cystic varieties. They are rarer in virgins (5%) than in multiparae (15%). Our 44 agent-free DBAb animals with mammary carcinoma showed tumors of the same acinar structure, with solid and cystic varieties similar to those of the DBA strain. However, they constitute only 50 percent of the total number of DBAb tumors (average age, 17 months). The other 50 percent included the following deviating types: *a*) Nine carcinomas with marked squamous metaplasia and keratinization in addition to acinar adenocarcinomatous or solid epithelial proliferation (average age, 20 months) (fig. 1). *b*) Five carcinomas with papillary epithelial proliferation (average age, 18 months) (fig. 2). *c*) Five tubulo-adenocarcinomatous forms, sometimes with solid parts (average age, 16 months) (fig. 3). *d*) Four animals showed other deviating forms of carcinoma (average age, 20 months). Two were solid large-cell carcinomas with marked dissolution of cells. The other two (one solid and one adenocystic) showed an abnormally cellular stroma with irregular cells resembling carcinosarcoma. These irregular stromalike cells were considered to be carcinoma cells (fig. 4).

These deviating types of tumor tissue correspond to the deviations of tumors in strain DBA mice described above. Several of these tumors were transplanted subcutaneously in series into young DBAb animals or their hybrids. During the course of 6 to 12 passages the typical structural characteristics remained intact. Later, however, loss of differentiation occurred; the final picture was that of solid carcinoma without characteristic structures. Since we transplanted into young animals, and since the tumors grew more rapidly after a number of passages, the loss of differentiation was considered to be related to the more rapid development.

Kirschbaum (*17*) suggested that mammary carcinoma in agent-free animals may in some cases be differentiated from carcinoma in animals with the agent on the basis of very marked squamous metaplasia. This is not the case in our DBA strain. The number of tumors with marked squamous metaplasia was relatively higher in DBAb animals; equally marked squamatization, however, occurred in the DBA line. In general, all forms of mammary carcinoma found in DBAb females also occurred in DBA females. The structure of a mammary tumor, therefore, does not indicate the presence or absence of the agent. It was found that DBAb mice without the agent show a wider variety of histologic pictures, and that the percentage of tumors with an acinar solid structure and its varieties is not so predominant as in animals with the agent. This corresponds to the findings of Heston, Deringer, Dunn, and Levillain (*5*) in C3H mice and, to a certain extent, also with the findings of Andervont and Dunn (*7*) who made comparative studies on mammary tumors follow-

ing treatment with methylcholanthrene in DBA mice with and without the agent.

Carcinoma develops later in DBAb than in DBA mice. However, this fails to explain the wider histologic variety of tumors, for old DBA animals (older than 17 months) also show a higher percentage of carcinomas with acinar and related structures, and a less wide variety than DBAb mice.

It is notable that the absolute number of carcinomas having variable form in DBA (a few of a large number of tumors) differs little from that in DBAb (many of a small number of tumors).

Other Tumors in Females of the DBAb Line

Fibrosarcoma of the uterus.—In addition to mammary tumors, fibrosarcoma of the uterus was the tumor most frequently observed in the DBAb line. It was found in 22 of 473 females older than 18 months. The age limit of 18 months was selected, since no sarcoma of the uterus was found under this age. The earliest tumor age was 496 days. The distribution of these tumors in virgins, breeders, and force-bred females is given in table 6. In these three groups, no significant difference in frequency was found.

Table 6.—*Uterine sarcoma in DBAb females older than 18 months*

	Total number of mice	Number of mice bearing uterine sarcoma	Average tumor age (*months*)
Virgins	91	3	20
Breeders	158	11	21
Force-bred	224	8	19

Sarcoma of the uterus was found in one of the three original females of the DBAb line. Excessive numbers of tumors in one of the 3 lines or in certain families was not observed. This tumor is not multiple; it may be localized in the cornua, or in the undivided part of the uterus. Metastases in the liver are frequent.

Carcinoma of the skin.—Three DBAb animals (2 virgins aged 19 and 22, and 1 breeder aged 18 months) developed an ulcerating tumor of the skin, consisting of markedly keratinized squamous epithelium, which involved the epidermis at the periphery. Although the tumors developed in the mammary region, there is nothing to suggest that they originated in mammary tissue. The picture corresponded with that of carcinoma of the skin.

Leukemia.—The frequency of leukemia in the females of the DBAb line was extremely low. It occurred in only 20 of 532 females older than 10 months. No difference between virgins and breeders was found. The average age was 512 days. No further analysis of the leukemia was made.

Other tumors.—One ovarian tumor (granulosa-cell tumor) was found in a breeding female, aged 21 months.

Hyperplastic Nodules in the Mammary Gland

Gardner (*18*) found that hyperplastic nodules occur in the mammary glandular tissue of high-cancer-strain female mice and that mammary carcinoma arises from such adenomatous foci. It is beyond doubt that these nodules are often absent from agent-free mice [Huseby and Bittner (*2*)]. However, they were recently demonstrated in old agent-free C3H mice, in 3 percent of which mammary carcinoma occurred [Jones (*19*)].

Since mammary carcinoma also occurred in our agent-free DBAb line, a study was made of the occurrence of nodules. In general, the 2nd and 3rd pair of mammary glands were fixed, dissected out, stained, and examined through a binocular loupe. One or more mammary glands were then cleaned and mounted; pathologically altered regions in other mammary glands were removed and examined in section. Whole mounts of the mammary glands of 171 mice of the DBA and DBAb lines were studied. These belonged chiefly to the series listed in table 1. In 50 percent of the cases, one 2nd-pair mammary gland was mounted; in the remaining cases, the other 2nd mammary gland, and/or one 3rd gland was also mounted. The nodules in these whole mounts were counted. When 2 or more mammary glands were enclosed, the highest number of nodules occurring in a single mammary gland was recorded, in order to keep the numbers comparable with the glands of those animals of which only the mammary gland with the most marked changes was mounted. The number of nodules in table 7 is therefore generally that of the mammary gland showing the most marked change.

Nodules were actually also found in our agent-free animals. They consisted of local hyperplasia of glandular tissue, *i. e.*, a focal accumulation of acini. The outermost acini often showed marked dilatation. In a number of foci, especially in older breeders and force-breeders of the DBAb line, a mononuclear infiltrate was found. These foci as a rule contained a number of acini with squamous epithelium. The structural arrangement of the acini themselves was similar to that of hyperplastic nodules without infiltrate. In a few respects, therefore, these nodules differ from those found in DBA females.

In our opinion, the mild lymphocytic and histiocytic infiltration in the nodules of the DBAb females is correlated with the involution of mammary tissue and suggestive of regression in the hyperplastic focus. The higher frequency of this infiltration in DBAb females is probably attributable to the fact that this group comprises a larger number of very old animals than the DBA group.

The infiltration and squamous metaplasia were considerably less marked than those found by Huseby and Bittner (*2*) in inflammatory nodules not regarded as a precancerous condition. Only a few nodules were found in which the infiltrate was so large that the nodule appeared

TABLE 7.—*Number of hyperplastic nodules per mammary gland in DBA and DBAb females of different ages*

30 DBA virgin mice																									
Age (*months*)	7	7	7	7	8	8	8	8	9	11	11	11	11	11	12	14	14	15	15	16	16	19	19	20	21
Nodules	0	0	0	1	0	1	1	2	0	0	3	2	3	0	4	10	diff.	8	10	9	13	diff.	diff.	2	5
Mammary Tumors	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	+	+	+	—
Age	21	21	22	22	23																				
Nodules	2	13	3	6	9																				
Tumors	—	+	—	—	+																				
18 DBA breeders																									
Age	6	8	9	9	9	10	12	12	13	13	15	19	20	20	20	21	22	22							
Nodules	0	9	0	8	0	0	0	8	6	14	5	0	7	8	5	3	1	3							
Tumors	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—							
47 DBAb virgins																									
Age	6	6	6	6	6	7	7	7	7	7	8	8	9	10	11	12	12	12	14	14	14	15	15	16	16
Nodules	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	diff.
Tumors	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Age	19	20	21	23	23	24	24	24	24	24	24	24	24	24	24	25	25	25	25	25	25	26			
Nodules	3	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	4	0	0	0			
Tumors	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—			
49 DBAb breeders																									
Age	9	12	12	12	12	12	12	12	12	12	12	12	12	13	13	13	13	13	15	15	15	18	19	19	19
Nodules	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	4	0	2	diff.	2	0
Tumors	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+	—
Age	20	20	21	21	21	21	22	22	22	22	22	23	23	23	23	23	23	23	23	23	24	24	25	26	
Nodules	0	0	1	2	3	1	1	2	diff.	1	1	0	5	2	2	0	0	1	2	0	4	5	0	1	
Tumors	—	—	—	—	+	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	+	—	—	
27 DBAb force-bred																									
Age	11	12	15	16	16	16	18	18	18	18	19	19	19	20	20	20	20	20	20	21	21	21	21	21	22
Nodules	5	diff.	diff.	5	7	4	diff.	3	4	11	2	4	4	7	4	4	4	3	0	8	3	2	0	0	4
Tumors	+	+	+	+	+	+	—	+	+	+	+	—	+	+	—	+	+	+	—	+	—	+	—	+	+
Age	24	24																							
Nodules	0	1																							
Tumors	+	—																							

to have originated from inflammatory foci instead of from local hyperplastic foci. These inflammatory foci were excluded.

Essential differences between the nodules in animals with the agent and those of agent-free animals were not observed.

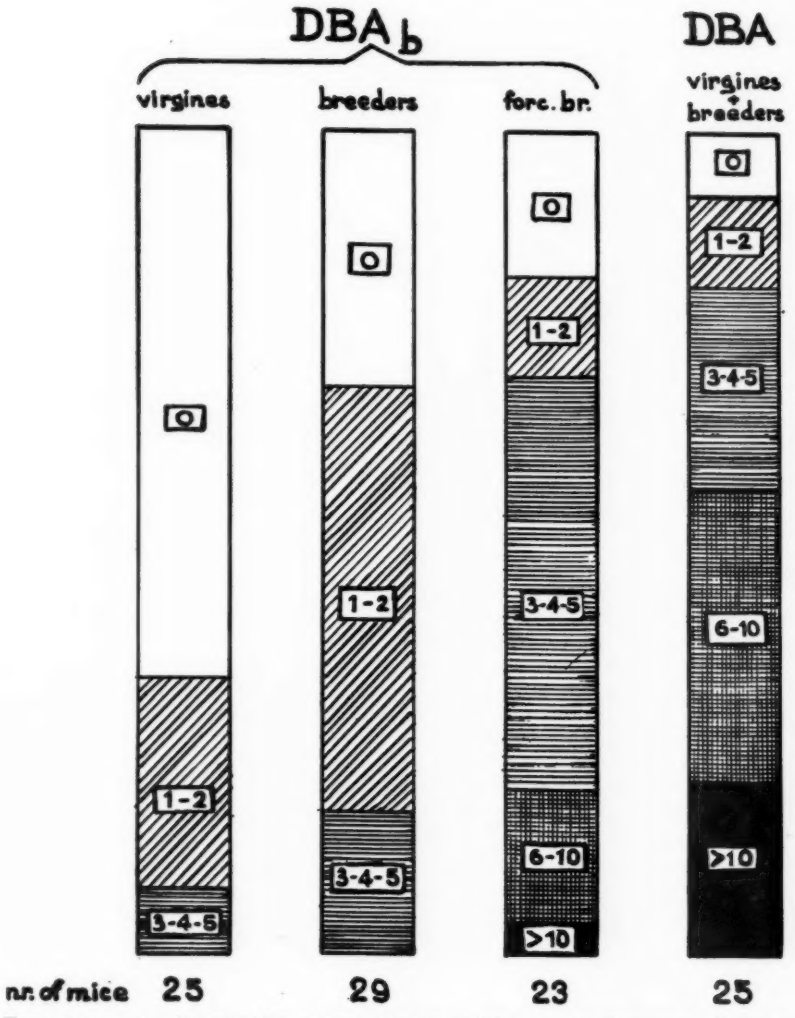
In a number of animals, the abundance of small hyperplastic regions precluded satisfactory counts. These cases were recorded as diffuse hyperplasia; the majority of the animals had mammary carcinoma. They were not included in the computation of averages. A survey of the animals examined is given in table 7, which shows that the groups are not directly comparable due to age differences. Comparative studies on the frequency of hyperplastic nodules should naturally be carried out on animals above a certain age limit. Table 7 shows that nodules are regularly found in DBA mice, aged about one year; in DBAb virgin and breeder mice, the minimum age proves to be 15 months. In order to ascertain the time at which the nodules develop in DBAb animals, all 10 mammary glands of 8 virgins and 8 breeders (12 to 16 months of age) were examined. A nodule was found in a mammary gland in only 1 virgin and 3 breeders. This shows that, in the absence of the agent, the nodules occur later. This corresponds to the findings of Jones (*19*), who observed nodules in agent-free C3Hb mice, but only in older animals. Huseby and Bittner (*2*) only rarely observed nodules in their agent-free C3H and A strain mice; however, they examined only a few animals older than 15 months.

Text-figure 4 shows the number of animals of the present experiment above these age limits in each group possessing 1 or 2, 3 to 5, 6 to 10, 11, or more, or no nodules. It clearly shows that in DBAb females both the number of animals with nodules and the number of nodules increases from virgins to breeders and from breeders to force-breeders. In DBA female mice, the frequency was much higher (here, virgins and breeders were combined, as the numbers were practically equal).

Table 7 shows that the animals with mammary carcinoma had a higher average of nodules than the tumor-free animals of the same group. The groups can be compared only after a correction: the number of carcinoma-bearing animals in each group should correspond to the relevant percentage of carcinomas given in table 2. After this correction, the frequency of nodules per group can be established.

Comparison of carcinoma-free animals exclusively is unjustifiable. In this manner, animals having many nodules would be eliminated; moreover, more animals would be eliminated in an agent-bearing than in an agent-free group.

DBA virgins.—There were 16 animals older than a year; all had 2 to 13 nodules. Diffuse hyperplasia was found in 3 of the mice. The remaining 13 mice had an average of 7 nodules (the figures of the average number of nodules denote the highest number found in one mammary gland). This group comprised 11 animals bearing mammary tumors (average number of nodules, 9) and 5 non-tumor-bearing animals (average, 4). Since the



TEXT-FIGURE 4.—Distribution of hyperplastic nodules in the mammary glands in DBAb and DBA female mice tabulated in table 7. In DBAb mice older than 15 months, the majority of the virgins had no nodules, most of the breeders have one or two nodules per gland, and most of force-breeders have three to five nodules. In DBA mice, older than 12 months, animals with 6 to 10 nodules are the most numerous.

percentage of carcinoma-bearing animals of this group corresponds approximately to the percentage of DBA virgins in general, correction is unnecessary; the average number of nodules can be established to be 7.

DBA breeders.—Ten of 12 animals older than one year possessed 1 to 14 nodules. The average number was 5. Only one animal had mammary

carcinoma (also 8 nodules). This group, therefore, comprises too few carcinoma-bearing animals. The average number of nodules in animals developing spontaneous carcinoma may be expected to be higher than that of animals which grew old without developing carcinoma. This is affirmed by the findings in the former group. The average number of nodules in our DBA breeders is therefore certainly higher than that shown by our group. Consequently, the number 5 should be corrected. The carcinoma-bearing virgin DBA mice possessed an average of 9 nodules; the non-carcinoma-bearing mice had four nodules. The carcinoma-free breeders had 5. The average number of nodules in carcinoma-bearing breeder mice may be expected to be at least as high as that of carcinoma-bearing virgin mice, especially since non-carcinoma-bearing breeders also have a slightly higher number of nodules than carcinoma-free virgins. In carcinoma-bearing DBA breeders, the average number of nodules may be expected to be 9 to 10. Since two-thirds of the breeders develop mammary carcinoma (table 2), the average number of nodules in a representative group of DBA breeders may be estimated to be 8 (instead of the 5 of our selected group).

DBAb virgins.—Ten of 26 animals older than 15 months possessed one to four (1 × diffuse) nodules. The average number for the entire group is 0.5. Three animals had carcinoma. This percentage is slightly too high; however, the average number of nodules for the entire group will not be considerably altered by this fact.

DBAb breeders.—Two of 31 animals older than 15 months had diffuse hyperplasia; of the remaining 29 mice, 20 had 1 to 5 nodules. Apart from the 2 with diffuse hyperplasia, the 29 animals had an average of 1.7 nodules (in one 2nd mammary gland). This group, however, comprised 10 with mammary carcinoma and an average of 2.3 nodules (1 × diffuse). The number of carcinoma-bearing animals, therefore, was relatively too high. The 21 animals without tumors had an average of 1 nodule. The total average in this group is too high, due to too high a percentage of carcinoma-bearing animals; after correction of this percentage to 12 percent (see table 2), the average number of nodules would be 1.2.

DBAb force-breeders.—Nineteen of 25 DBAb mice older than 15 months possessed 1 to 11 nodules; 2 had diffuse hyperplasia. The average number of nodules was 3.3. However, the number of carcinoma-bearing animals in this group was much too high, namely 17, with an average of 4 nodules (1 × diffuse). The 8 tumor-free animals had an average of 1.7 nodules (1 × diffuse). When the percentage of carcinoma-bearing animals is corrected to 16 percent (table 2), the average number of nodules in this group is 2.1.

The frequency figures for hyperplastic nodules are therefore: DBAb virgins, 0.5; DBAb breeders, 1.2; DBAb force-breeders, 2.1; DBA virgins, 7; and DBA breeders, 8.

This warrants the conclusion that the mammary tumor agent exerts considerable influence on the development of hyperplastic nodules. The

number of nodules of DBA virgins is 14 times as high as that of DBAb virgins; that of DBA breeders is 7 times as high as that of DBAb breeders. The difference between virgins and breeders expresses the influence of pregnancy on the development of nodules. In DBA mice, this difference is not important; it is much more marked in DBAb mice for the breeders have at least twice as many nodules as virgins, and force-breeders nearly twice as many as breeders.

In our opinion, the fact that the influence of pregnancy is much less marked in DBA than in DBAb mice should be attributed to the considerable influence of the agent, due to which so many nodules develop in DBA virgins that this number is not very much increased by the influence of pregnancy.

Relationship Between Hyperplastic Nodules and Carcinoma

Hyperplastic nodules are generally considered to represent a precancerous condition. Remains of a nodule are frequently found at the periphery of carcinomas and early carcinomas are found in sections of nodules. To a certain extent, the nodules are autonomous, and do not react to normal influences in the same manner as does normal mammary tissue. They may remain unaltered, for instance, while the remaining mammary tissue shows involution. They may persist for a long time. Old animals show nodules in the same number as do animals aged 1 to 1½ years (see table 7). On the other hand, they may very well disappear. In some cases, localized accumulation of pigment with remains of epithelial tissue were found in the vicinity of the galactophorous ducts; they were regarded as remains of nodules which had regressed. The mononuclear infiltrate sometimes found in nodules in older animals is probably also correlated with regression of the hyperplastic tissue.

Hyperplastic nodules and carcinomas were also observed in our agent-free animals of the DBAb line. The frequency of nodules and carcinomas is markedly increased in the presence of the agent. The carcinogenic influence of the mammary tumor agent may be twofold: *a*) The mammary tumor agent causes the development of more nodules, and the risk of development of carcinoma is proportionally increased. *b*) The mammary tumor agent is associated with the development of more nodules, and these nodules show a more marked tendency to develop into carcinoma than they would in the absence of the agent.

In our experimental series, the frequency of nodules proved to be completely parallel to that of carcinomas (see table 8). In the presence of the agent, virgin mice show a high frequency of nodules, being increased 14 times, and the frequency of carcinoma was increased 15 times. In breeders, the frequency of both is increased 6 to 7 times by the agent. In agent-free animals, the frequency of nodules is increased 2½ times by breeding and 4 times by forced breeding, while that of carcinoma is increased 3 and 4 times, respectively. In DBA animals with the agent, the frequency of carcinoma is slightly increased by breeding (1.2 times), the estimated

increase in the frequency of nodules being equally unimportant. Consequently, the carcinoma percentage would seem to be constantly proportional to the frequency of nodules. Of a certain number of nodules, in other words, invariably the same percentage becomes carcinomatous during the time they exist in DBA mice, whether they be virgins, breeders, force-breeders, agent-free animals, or agent-bearing mice.

TABLE 8.—*Frequency of hyperplastic nodules compared with mammary cancer incidence*

	DBAb			DBA	
	Virgins	Breeders	Force-breeders	Virgins	Breeders
Ratio of occurrence of nodules.....	0.5	1.2	2.1	7	(8)
Percentage of mammary cancer.....	4	12	15	59	68

It should be borne in mind, however, that the remaining nodules in animals succumbing to carcinoma cannot possibly develop into carcinoma. This is of importance especially in the DBA groups. On the other hand, the nodules in DBAb animals develop later and may therefore have less time to develop into carcinoma.

Our figures suggest that the influence of the agent, and of breeding and force-breeding, on the tumor frequency consists exclusively of an increase in the number of hyperplastic nodules and not of direct influences favorable to the development of adenomatous hyperplasia into progressive carcinoma.

DISCUSSION

Transmission of the mammary tumor agent in high-cancer strains of mice takes place via the mother's milk. Mice removed from the uterus of pregnant animals of high-cancer strains shortly before birth, and nursed by foster mothers belonging to an agent-free strain, do not receive the mammary tumor agent. This method is therefore used in starting agent-free lines of high-cancer strains. The method is only reliable, however, if intra-uterine transmission of the agent from the mother to the young is excluded with certainty. The following possibilities of prenatal transmission are taken into account: *a) the mammary tumor agent may be contained in the cytoplasm of the ovum; b) the agent may be transmitted to the ovum by the sperm, either during fecundation or by absorption of the agent from the milieu surrounding the ovum before implantation; c) the agent is transmitted from the mother to the young via the placenta.*

No evidence of transmission of the agent by one of the above-mentioned methods has hitherto been found. On the contrary, experience suggests that intra-uterine transmission of the agent does not take place. This view is supported and substantiated by the results of the present experiments. No agent was demonstrated in extracts of the organs of embryos from the uterus of high-cancer-strain females, injected into sensitive test

animals. The agent was demonstrated, however, in similar extracts of maternal tissue.

Negative test findings may be attributable to the fact that the agent is present in the embryos, but only in minimum quantities not demonstrable by the methods used. Since the agent has not yet been isolated and characterized, its presence or absence can only be judged by its actions. The agent has two characteristic properties: *a*) its multiplication in living animals, and *b*) its transmission via the mother's milk.

If the embryo *in utero* acquires a small quantity of the agent, this quantity will increase during the animal's lifetime and its offspring will acquire a larger quantity with the mother's milk. In this manner, another strain with a high frequency of mammary tumors would develop after a few generations. Analysis of 7 agent-free generations of the DBAb strain reported here shows that this is not the case. Mammary tumors were found, but not in excessive numbers in certain families. Moreover, the test for the presence of the agent in these tumors was negative. This warrants the conclusion that intra-uterine transmission of the agent to offspring does not take place. Another conclusion is that mice may develop mammary tumors without the mammary tumor agent. It is noteworthy that there is no essential difference as regards structural characteristics between agent-free mammary tumors and mammary tumors of the same strain with the agent. However, the agent-free tumors show a greater variability of histologic form.

It is generally agreed that hyperplastic nodules occur in the mammary glands of high-cancer-strain females and that mammary carcinoma arises from such adenomatous foci. Examination of whole mounts of the mammary glands of agent-free DBAb animals showed that similar nodules occur in agent-free animals. The pathogenic mechanism of mammary carcinoma in animals with and without the agent may therefore be presumed to be the same. Further analysis of the number of hyperplastic nodules showed that the mammary tumor agent exerts a considerable influence upon the development of these nodules. It further suggests that the agent should be regarded as an accelerator and intensifier of developments in the mammary gland, which would also take place in the absence of the agent. It is likely that the action of the mammary tumor agent consists exclusively of an increase in the number of hyperplastic nodules and their earlier occurrence, and not of direct influence favorable to the transformation of adenomatous hyperplastic tissue into carcinomatous tissue.

A similar accelerating action is observed under the influence of pregnancy, *i. e.*, representing probably a hormonal influence. In the agent-free DBAb strain there is a significant difference in the tumor rate, and therefore in the number of hyperplastic nodules, between the virgins and the multiparae. This similarity between the action of the agent and that of hormones does not necessarily imply that the agent exerts its influence via the hormonal system. However, the possibility does

exist. Numerous investigations so far carried out in order to demonstrate a possible effect of the agent upon the endocrine system have failed to lead to a generally accepted conclusion.

SUMMARY

Shortly before birth, the young of a female high-cancer-strain Dilute-brown (DBA) mouse were operatively removed from the uterus and then nursed by a foster mother belonging to the low-tumor strain C57BL. These young—3 females and 1 male—were used to start a line called DBAb, of which 442 females of the first 7 generations were observed. Ninety-six of these females were kept as virgins; they showed a mammary tumor incidence of 4 percent, with an average tumor age of 21.6 months. Sixty females were breeders with only 1 litter; this group showed only one mammary carcinoma (at 18 months). Of 120 females with an average of 6 pregnancies, 12 percent developed mammary carcinomas at an average age of 18.8 months. Of 166 females that were force-bred, 31 had only one litter, and 16 percent developed mammary carcinomas at the average age of 21 months. The remaining 135 force-bred females had an average of 7 litters; 15 percent developed mammary carcinoma at an average age of 16.7 months. The following results suggest that the mammary tumors in the DBAb line developed in the absence of the mammary tumor agent: *a*) The mammary tumors show no familial accumulation; the pedigree charts show that the tumors are distributed at random. *b*) No agent could be demonstrated in extracts of mammary tumors in the DBAb line, while comparable tests on tumors of the DBA line were positive. *c*) Extracts of the organs of embryos removed from the uterus of DBA females shortly before birth revealed no tumor agent, though this was found in the mother.

The results do not substantiate the theory of "infection" of the young with the tumor agent *in utero* (in the high-cancer strain DBA).

The morphologic structure of the mammary tumors in the agent-free DBAb line shows no essential difference from that of the mammary tumors in the DBA strain bearing the mammary tumor agent. However, the agent-free mammary tumors show a higher variability in histologic aspects.

A comparative study was made of whole mounts of the mammary glands of the DBA line bearing the agent, and of the agent-free DBAb line. Hyperplastic nodules were also found in DBAb females; however, they are less in number than in DBA females and occur at a later age. Analysis of the number of nodules and their relationship to the number of carcinomas warrants the conclusion that the effect of the agent probably concerns exclusively the formation of hyperplastic nodules and not the transformation of these nodules into carcinomas.

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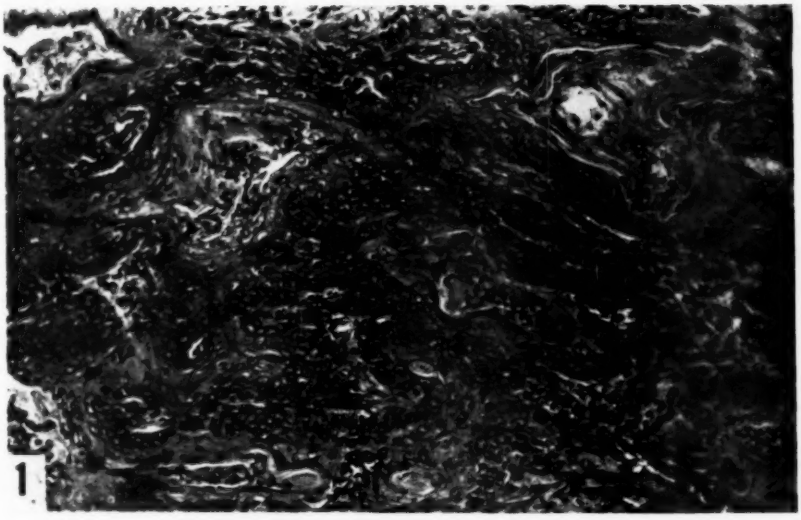
PLATE 57

FIGURE 1.—Mammary carcinoma with extensive squamous metaplasia and keratinization. DBAb breeder, 17 months. × 148

FIGURE 2.—Adenocystic mammary carcinoma with papillary structure. DBAb force-bred, 12 months. × 350



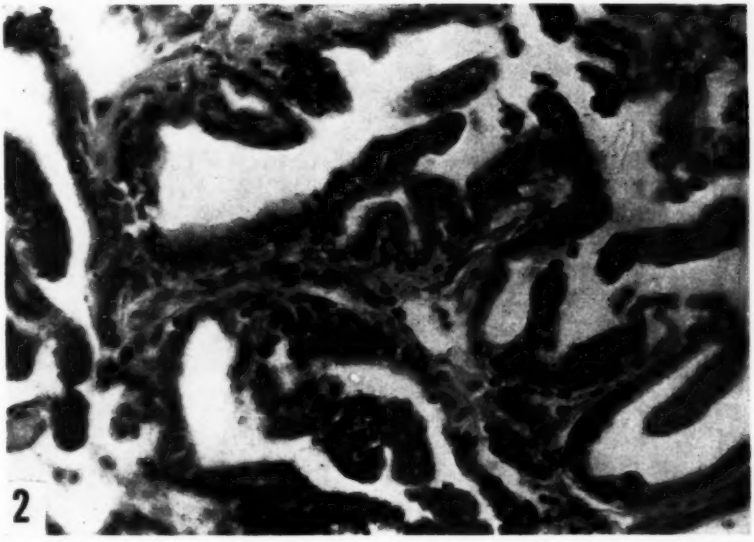


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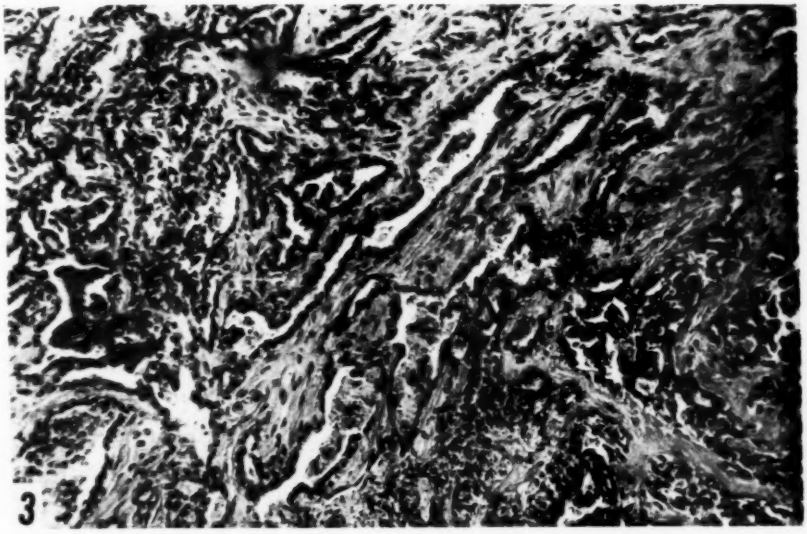
PLATE 58

FIGURE 3.—Tubulo-adenocarcinoma of mammary gland. Tumor cells form clefts and tubules, surrounded by compact stroma. DBAb breeder, 18 months. × 148

FIGURE 4.—Solid mammary carcinoma showing invasion of stroma by tumor cells. Stroma abnormally cellular. This picture corresponds to so-called carcinosarcoma. DBAb force-bred, 13 months. × 350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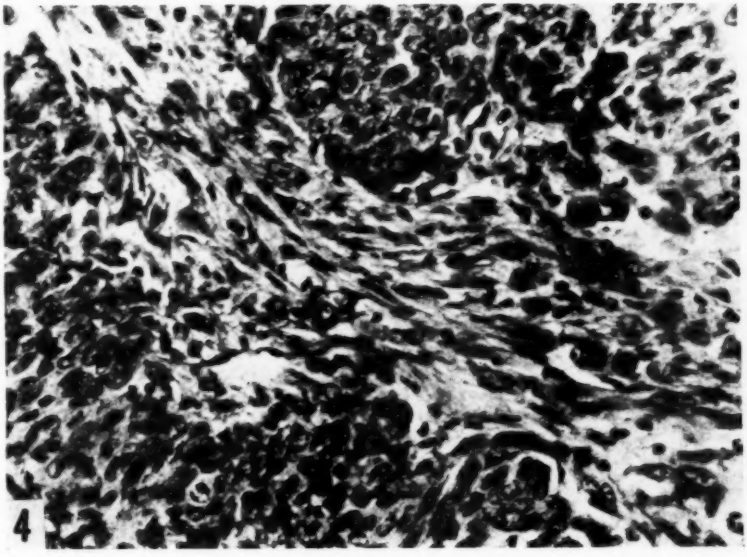
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Variation in Infectivity and Virus-Particle Content of Individual Plasmas from Birds with Erythromyeloblastic Leukosis [1, 2]

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Erythromyeloblastic leukosis, like other virus-induced forms (*1–3*) of the fowl leukosis complex, may be readily transmissible by means of cell-free filtrates (*4, 5*). Nevertheless, wide variations are observed (*6, 7*) frequently in the quantitative aspects of the infectious characters of such materials. Some of the factors influencing the fluctuation are evidently associated with the age of the recipient hosts and the natural states of resistance and susceptibility of the recipient host population. It has been suggested (*1*), also, that variation may be associated with differences in the strains of the virus and that virulence may be influenced by the age of the donor bird. Efforts have been made (*7*) to increase and stabilize the level of infectiousness by frequently repeated passage of the virus.

Attention was drawn to the phenomenon in this laboratory in recent work on quantitative titration (*5*) and purification (*8, 9*) of the erythromyeloblastic leukosis virus. Whereas, in most of these studies on titration, high infectivity and relatively little variation were observed (*5*), very great differences were seen in the infectivity of the various pools of starting materials and centrifugal fractions of them in the purification experiments, and low infectivity was observed more often than high levels of virulence. A clue to the cause of these results was the observation that the small amounts of plasma needed for the titration experiments were usually taken from young donors, while the larger pools needed for the centrifuge experiments were drawn from the older, as well as the younger birds. Since the success of the purification procedure is depend-

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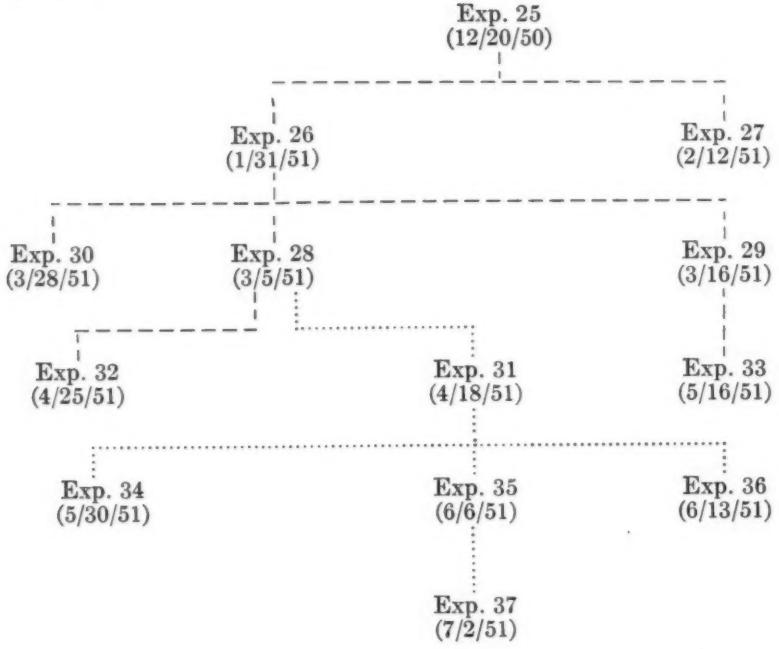
[3] Research Fellow of the National Cancer Institute, U. S. Public Health Service.

ent on obtaining adequate quantities of infectious virus, an investigation was made to learn whether there might actually be a significant dependence of the infectivity of the plasma of individual donors with erythromyeloblastic leukosis on the length of time between innoculation with virus and the time of bleeding. In addition, direct counts were made by ultracentrifugal and electron microscopic procedures on the number of virus particles present in the plasma samples. The results of these studies are described in the present report.

MATERIALS AND METHODS

The condition studied was erythromyeloblastic leukosis (*10*), BAI strain A, originally isolated by Hall, Bean and Pollard (*7*) and brought to this laboratory by Dr. E. P. Johnson. Numerous subsequent passages of the disease have been made in work already reported (*5*) in the course of the past 2 years. The interrelationship of the various passages and the derivation of the plasma specimens of the present work are shown in Chart 1, which is a continuation of Chart 1 of (*5*).

Chart 1. Interrelations of material used in the present experiments. This chart is a continuation of Chart 1 of (*5*) showing the interrelations in previous experiments. All of the passages indicated by broken dash lines (– –) were made with filtered plasma, while those indicated by the dotted lines (. . .) were made with plasma freed of cells by centrifugation (see text).

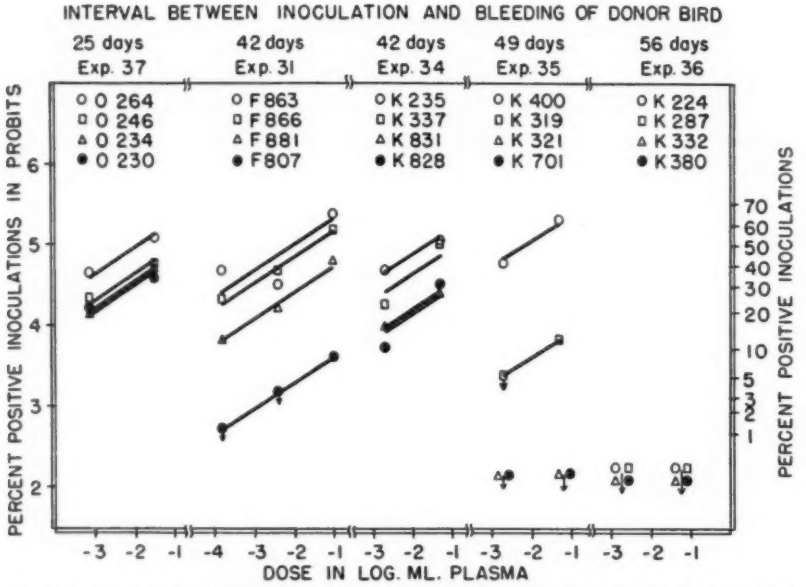


Individual donors for the studies were chosen from various titration experiments which happened to be under way at the time. All of these birds had been inoculated, as previously described (*5*), 3 days after hatching. Choice of the donors from these groups for the present work was made principally on the basis of the interval between their inoculation and the time at which the bird showed characteristic evidence of disease as seen in routine blood smears taken twice each week. On the day before the experiment, small amounts of blood were drawn from prospective donors for blood counts and hematocrit examinations. Early on the morning of the experiment, the selected donors, which had been fasted for about 24 hours, were bled from the heart into heparin (Liquaemin, 'Roche-Organon', 1 ml. = 10 mg.) in the ratio of 1 ml. heparin to 9 ml. blood. Counts of the primitive cells were made in Neubauer chambers with phloxine solution as diluent (*5*). The cells were removed by spinning the blood twice in round-bottomed tubes in the horizontal centrifuge at 2,000 × g for 15 minutes in the cold. Each plasma was then spun a third time in 15 ml. glass tubes with conical bottoms. No sediment was visible, but the plasma was removed with great care, leaving about 1 ml. at the bottom of the tube.[4] All procedures were carried out as rapidly as possible and, except for the time for preparing the dilutions, the materials were kept in the refrigerator or in vessels immersed in ice water. No more than 2 to 4 hours elapsed between bleeding and final injection of the materials.

Estimates of the level of virus infectivity of the various plasmas were made on the basis of quantitative dose-response relationships revealed in work previously reported (*5*). The test plasmas were inoculated undiluted or in dilutions made in the appropriate range with BSA-Simms' solution, as indicated in table 1 and text-figure 1. This solution consisted of 1.5 percent bovine serum albumin (5.0 ml. of the 30 percent solution of bovine serum albumin, Armour, added to 95 ml. of the saline solution) in Simms' (*11*) salt solution. All inoculations were made, in 0.1 ml. volumes, intravenously into 3-day-old New Hampshire chicks. Each dilution of each plasma was injected into 3 dose-groups of 60 (experiment 31) or 2 dose-groups of 90 chicks each (experiments 34, 35, 36, 37), a total of 180 chicks per plasma. All of the five experiments described here were made with chicks hatched from eggs of the same flock of hens and over a period of 3 months. Beginning 15 to 17 days after inoculation, smears were taken from each bird twice weekly for a period of 7 weeks.

In all of the experiments except one (experiment 31), counts were made of the number of virus particles in each plasma by a method developed (*9*) during the course of the studies. For this procedure, which has been employed also in previous work and will be described in detail in a subsequent report, the plasma was diluted in the range of 1–10 to 1–50. The plasma preparation was placed in a specially constructed ultracentrifuge rotor cell (*12*) containing, at the bottom, a thin block of agar, and the

[4] The plasma in each case was too small for filtration.



TEXT-FIGURE 1.—Dose-response relations obtained with the plasmas of individual chickens with erythromyeloblastic leukosis. The numbers in the body of the figure signify the donors of the respective plasmas, and the arrows indicate the inoculums which gave no response.

TABLE 1.—*Response of test chickens to graded doses of plasma from 20 individual donor birds obtained at various intervals after inoculation with the virus of erythromyeloblastic leukosis*

Experiment No.	Donor	Interval after inoculation (days)	Incidence of positive inoculations (dose in log ml. plasma)						
			−1.0	−1.3	−1.52	−2.4	−2.7	−3.13	−3.8
31	F 863	42	38/58	.....	.....	17/55	.....	.....	20/53
	F 866		34/59	.....	.....	20/54	.....	.....	15/60
	F 881		25/59	.....	.....	12/56	.....	.....	6/55
	F 807		5/60	.....	.....	0/60	.....	.....	0/60
34	K 235	42	.....	47/89	.....	.....	32/84	.....	.....
	K 337		.....	44/87	.....	.....	20/87	.....	.....
	K 831		.....	23/84	.....	.....	13/82	.....	.....
	K 828		.....	27/86	.....	.....	9/87	.....	.....
35	K 400	49	.....	55/86	.....	.....	35/86	.....	.....
	K 319		.....	10/83	.....	.....	0/85	.....	.....
	K 321		.....	0/82	.....	.....	0/85	.....	.....
	K 701		.....	0/88	.....	.....	0/88	.....	.....
36	K 224	56	.....	0/76	.....	.....	0/87	.....	.....
	K 287		.....	0/87	.....	.....	0/89	.....	.....
	K 332		.....	0/87	.....	.....	0/83	.....	.....
	K 380		.....	0/88	.....	.....	0/83	.....	.....
37	O 264	25	.....	.....	46/86	.....	.....	32/88	.....
	O 246		.....	.....	34/89	.....	.....	22/87	.....
	O 234		.....	.....	32/87	.....	.....	17/87	.....
	O 230		.....	.....	30/88	.....	.....	19/87	.....

TABLE 2.—*Summary of the data of the five experiments*

Experiment No.	Donor chicken No.	IIB* (*days*)	IPB† (*days*)	Primitive cell count per ml. ($\times 10^7$)	Log relative infectivity (*M*)	Standard error of *M*	Virus particles per ml. ($\times 10^9$)
31	F 863	42	2	42	+0.075	±1.103	.....
	F 866	42	12	68	−0.377	±0.431	.....
	F 881	42	16	92	−1.770	±0.479	.....
	F 807	42	16	132	−5.220	±0.891	.....
34	K 235	42	8	53	−0.282	±0.424	1.03
	K 337	42	12	34	−1.010	±1.044	3.49
	K 831	42	12	70	−2.415	±0.500	4.97
	K 828	42	8	75	−2.539	±1.128	6.5
35	K 400	49	16	127	+0.213	±0.426	1.70
	K 319	49	23	135	−4.264	±0.723	1.88
	K 321	49	9	103	<-5.0	.....	1.97
	K 701	49	6	34	<-5.0	.....	1.92
36	K 224	56	2	10	<-5.0	.....	7.2
	K 287	56	21	110	<-5.0	.....	6.71
	K 332	56	4	53	<-5.0	.....	2.86
	K 380	56	2	22	<-5.0	.....	0.1
37	O 264	25	8	43	0	.....	2.69
	O 246	25	5	6	−1.114	±0.444	1.31
	O 234	25	5	11	−1.450	±0.460	3.22
	O 230	25	5	18	−1.462	±0.460	0.68

*Interval between inoculation and bleeding of donor bird.
†Interval between first positive blood smear and bleeding of donor bird.

virus particles were sedimented onto the agar surface at 16,400 × g for 30 minutes. The supernatant fluid was pipetted off and the agar surface washed with 3 changes of calcium-free Ringer's solution containing 1 percent heparin. The agar block was removed from the cell, the surface allowed to dry, and the virus was fixed (*9*) with osmic acid vapor. Collodion in 0.5 percent concentration in amyl acetate was pipetted onto the agar surface, the excess drained away, and the film, carrying the virus particles, was floated off onto the surface of distilled water. The film was picked up on the wire screen and shadowcast with chromium.

RESULTS

As seen from the data given in tables 1 and 2, the studies were made on donors bled 25, 42, 42, 49, and 56 days after inoculation with the material derived from the experiments shown in Chart 1. It is seen that, while the interval between inoculation and bleeding of the individual donors of a given experiment (and thus also the chronological age of the bird) was constant, there was some variation in the intervals during which the various donors of the experiment were positive before bleeding.

Analyses of the data for estimates of infectivity have been made on the basis of the previously determined (*5*) linear relationship of dose in ml. of plasma to incidence expressed in probits. The results of the inoculations given in table 1 are shown graphically in text-figure 1. Straight lines of a

common slope were drawn through the means of the respective groups of points obtained with the individual plasmas. Calculation of this slope was made by the method described by Finney (*13*) using only the results with those plasmas giving more than one experimental point. Inspection shows a very good fit in all instances in which two or three points were observed (omitting the results with F 807 and K 319), with the exception of those of donors F 863, K 337 and K 828. However, the χ^2 test for comparing the individual slopes with the common slope gave no significant variation in any instance.

No point of reference has yet been devised, such as the 50 percent point employed with many viruses, for comparing the levels of infectivity of different preparations containing the erythromyeloblastic leukosis virus. Moreover, because of instability of the virus, a standard preparation is not available as a control from one experiment to another. As an improvisation, in order to compare numerically the levels of infectivity of the individual plasmas in the present work, the findings with the plasma of a single donor, O 264 (table 2, text-fig. 1) have been used as a standard of reference for the entire series. The necessary calculations were made by the procedure of Bliss (*14*) for the determination of relative potencies and their errors. The values obtained, given in table 2, represent log relative infectivity, which is the log of the ratio between the potencies of a given plasma and plasma O 264. This procedure is directly applicable in experiment 37, in which plasma O 264 was actually injected. It is understood that the usefulness and accuracy of the values obtained in the calculations from the results of the other experiments are dependent on many factors in the conditions of the experiments, including possible differences in the state of resistance or susceptibility of the various host populations.

The results, shown graphically in text-figure 1, and the values of the relative infectivities given in table 2, reveal unequivocally a wide variation in the infectious capacities of the individual plasmas. In addition, it would appear that the degree of variation is related to the time at which plasma was taken after inoculation of the donor. While much variation is seen with plasmas taken in the intervals of 25 to 42 days, the differences are more extensive among the specimens of the 49-day interval. Plasma K 400 was of the highest order of infectivity (+0.213); K 319 of the lowest (−4.264), and plasmas K 321 and K 701 (<-5.0) caused no disease in the test hosts in the doses employed. None of the plasmas of the 56-day interval produced disease. The very broad range of the individual infectious capacities of the plasmas is emphasized in the numerical expressions of relative infectivity given in table 2. There was no evidence that the level of infectivity was related to the duration of the disease in the donor.

Calculations were made of the "average" levels of infectivity of the groups of plasmas in the various experiments. This was done by summing the antilogs of the individual log relative infectivities in the respective groups, dividing by four in each case and reconverting the values to logs.

The levels calculated in this way, in the order of the experiments of text-figure 1, were -0.410, -0.807, -0.389, -0.543 and <-5.0. These values, which were rather uniform in the first four instances, despite the wide variation among the individual plasmas, are probably close to those which might have been obtained from pools of aliquots of the plasmas in the individual experiments if dilution was the only factor influencing the infectivity of each plasma in the various pools. These average infectivity levels in four instances thus are closely comparable with those of the pools previously reported, as seen on comparison with text-figure 2 of (*5*). One possible pool, that of the plasmas of the 56-day interval, would have been entirely noninfectious.

The virus-particle content of the plasmas is given in table 2. The differences, with one exception (K 380), were within the range of a single log interval, and there was no relation between the values and the levels of plasma infectivity. It is evident, also, that no relation can be detected between the number of primitive cells and either the particle count or the infectivity of the corresponding plasmas.

DISCUSSION

The present experiments may be regarded as having demonstrated conclusively exceedingly wide differences in the infectious capacities of the plasmas of individual chickens with erythromyeloblastic leukosis. Furthermore, within the limits of the small series studied, certain aspects of these differences appear to be related to conditions associated with the length of the interval between the inoculation and bleeding of the donor bird. That the infectivity of plasma in erythromyeloblastic leukosis may vary, frequently to the complete loss of ability to transmit the disease, is well known (*6*, *7*). The quantitative aspects of the phenomenon and its relation to the individual chicken, however, have not been previously reported. It is noteworthy here that all of the studies were made within a brief period and that 12 of the plasmas exhibiting the whole range of variation were obtained from individuals all of which had received, initially, portions of the same inoculum.

It is evident that the results are entirely adequate for understanding at least one basis for the apparent fluctuations in the infectious capacity of the leukosis virus. As seen in text-figure 1, the virulence of a given specimen would depend greatly on the individual donor, and the likelihood of obtaining a highly infectious plasma greatly decreases with the increase in the interval between inoculation and harvest of the plasma. In judging the significance of the observed fluctuations, there should be borne in mind the conditions necessary for quantitative titration of the infectivity of the leukosis virus. For example, it is evident that the fluctuations observed by Hall *et al.* (*7*) can be explained readily on the basis of individual chicken variation and the variation to be expected through the use of small numbers of test hosts. There has been no evidence in this laboratory of any systematic increase or decrease in the virulence of the strain of virus under study over a period of 3 years.

Obviously, these few data can give no concept of the quantitative distribution of individual variations. It should be emphasized that the absence of infectivity in 2 birds of the 49-day and 4 of the 56-day interval does not indicate that such results will be encountered always in this proportion. Instead, the data show only that the longer the interval the more chance there is of obtaining a noninfectious plasma. That the trend is real and that this factor is of critical importance in selecting donors for transmission of the disease by plasma has been clearly shown in numerous experiments subsequent to the recognition of the time influence.

The results do not seem sufficient, however, to explain low or no infectivity of pools of mixtures of the plasmas taken from birds with short and others with long intervals after inoculation. Simple dilution of a highly infectious plasma with, say, equal volumes of 3 entirely noninfectious plasmas would hardly account for an entire lack of infectivity. Instead, it would seem necessary to assume the presence of some inactivating material in the noninfectious plasma capable of affecting the virus of the infectious plasma of the pool. The problem is further complicated by the finding of as many virus particles in the inactive plasmas as in the most infectious ones. This aspect of the data brings erythromyeloblastic leukosis into line with the chicken sarcoma and rabbit papillomatosis with respect to the occurrence of "masked" virus (*15*). The observations on leukosis differ greatly from those with the other two neoplastic states, for in leukosis the inactive virus particles have been enumerated directly in electron micrographs. In rabbit papillomatosis, masked virus is detected only by the induction of immune bodies in animals bearing papillomas from which active virus cannot be obtained. That the sarcoma virus seems masked, though persisting when it cannot be demonstrated by infectiousness, can be shown by the recurrence of infectivity following cell passage of the sarcoma under proper conditions. In neither of these conditions is anything known about the mass or the number of virus particles involved in the masking process.

The mechanism responsible for the masking of the leukosis virus is as obscure as that involved in the analogous phenomena associated with the chicken sarcoma and the rabbit papilloma virus. In those two instances, it has not been possible to relate neutralizing antibodies to virus inactivity (*15*). Only preliminary experiments have been made to learn if the noninfectious plasmas contain materials capable of neutralizing the leukosis virus of active plasmas *in vitro*. It might be suspected from the results, however, that while the noninfectious plasmas may contain antibody, some other explanation must be sought to account for the inactivity of the large number of virus particles encountered in the noninfectious plasmas and for the possible inactivation of active virus in pools. Whatever its nature, the factor in leukosis is associated with the interval following inoculation. Whether age *per se* or the time interval of experience of the donor with the virus is the principal contributor to

the inactivation is not evident now. Age is a condition influencing the activity of filtrates of the chicken sarcoma, but it is notable that the age range in that disease extends over periods of years, rather than a few weeks.

SUMMARY

Variation through the range of at least five $\log_{10}$ increments has been observed in the infectious capacities of plasmas from individual chickens with erythromyeloblastic leukosis. The differences are associated, at least in part, with the length of the interval between the inoculation and the bleeding of the donor bird. Within an interval of about 25 to 40 days infectious capacity, though variable, tends to be high; afterward, the variation is greater and the plasmas of a large proportion of the birds are noninfectious in the doses employed.

The number of virus particles in 20 of the 24 plasmas studied varied from 0.1×10^9 to 7.2×10^9 per ml., and the number of primitive cells from 6×10^7 to 135×10^7 per ml. No interrelation was observed between infectivity, number of virus particles, or the number of primitive cells. The basis for the individual variations in plasma infectivity and the occurrence of inactive virus in the plasmas remains obscure.

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Polysaccharide Synthesis in Frozen Tissue Sections as a Histochemical Method for Phosphorylase [1, 2]

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In a program of biochemical and histochemical studies of tissues of the human female genital tract, experiments on phosphorylase assays were undertaken as part of a search for possible aberrancies in carbohydrate metabolism in tumors of the uterus. For part of these studies, the histochemical demonstration of phosphorylase was desirable. A complete histochemical demonstration of phosphorylase activity should show both glycogen and phosphate ion after incubation with glucose-1-phosphate, under suitable conditions. This report deals with the conditions found to be most suitable for histochemical demonstration of polysaccharide synthesis and liberation of phosphate ion from glucose-1-phosphate in sections of frozen tissues. The liver of starved rats proved to be the most satisfactory test material employed, since it contains little glycogen and is known to be rich in phosphorylase. Studies with other tissues, human and rodent, yielded comparable results. A similar histochemical demonstration of glycogen synthesis has been attempted by Cobb (*1*), as reported by Bradfield (*2*). Cobb worked with sections of bone that had been prepared by the Altmann-Gersh frozen-dehydration technique prior to digestion and incubation. Yin and Sun (*3*) were able to demonstrate phosphorylase activity in fresh sections of plant material by formation of starch from glucose-1-phosphate.

METHODS

Tissues used in this study were the following: liver and kidney of the rat; uterus of castrated rabbits treated with estrone and/or progesterone; and human endometrium and cervix uteri. The rats included starved and fed, male and female, and young and old animals of a hooded strain

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inbred for 11 years at the Woman's Clinic of The Johns Hopkins Hospital. New Zealand White strain rabbits were used as the source for segments of the uterus. The rabbits were kindly donated to us from animals used in independent experiments by Dr. Arpad Csapo of the Department of Embryology of the Carnegie Institution. The human tissues were obtained by endometrial or cervical biopsy. Rats were killed by a blow on the head. Glycogen depletion in rat liver was effected by starvation for periods of 18 to 48 hours. Rabbits were anesthetized with nembutal. Patients were anesthetized by sodium pentothal, nitrous oxide, or ether. Pieces of tissue up to about 1 cm. in diameter were frozen as rapidly as possible in melting white gasoline or isopentane, which had previously been solidified by immersion in liquid nitrogen. Tissues were either cut at once in a cryostat kept at about −20° C., or stored at −30° C. until needed. A sliding microtome mounted in a Linderstrøm-Lang type cryostat was used to cut the material at 15 to 30 microns. Sections were flattened with a small brush on clean 1 × 3 inch glass slides (no adhesive being used), or placed directly in the incubation mixtures. The slides with adhering sections were stored in the cryostat (usually less than 1 hour) until ready for incubation, which was usually at 37° C., and in a few instances at 30° C.

Because of some inconsistency of results from day to day, even when the same piece of tissue was used, considerable effort was expended in attempting to achieve greater reproducibility by varying *p*H, composition of substrate, and temperature. Originally, a substrate used in the homogenate technique described by Sumner and Somers (*4*), based on the work of Cori *et al.* (*5*), was employed. On occasion, 0.036 M $CaCl_2$ was included in the substrate inasmuch as it was important to ascertain phosphate production also. Subsequently, various changes in the incubation medium were made. Finally, it was found that a rather simplified solution with the following composition was at least as good as any tried and actually gave the most consistent results obtained to date. The compounds incorporated into this medium were suggested to us by the work of Maengwyn-Davies *et al.* (*6*), and Sutherland *et al.* (*7*).

Distilled water	7.5 cc.
Glucose-1-phosphate (K_2 salt)	80 mg.
NaF	63 mg.
Saturated aqueous sodium acetate solution	7.5 cc.

Adjust to about *p*H 6.0 with a few drops of HCl.

*p*H measurements before and after incubation were made with a Beckmann glass electrode *p*H meter. Infrequently, a shift of as much as one *p*H unit to the acid side was encountered. In overnight incubations, heavy bacterial contamination was encountered at times in the relatively dilute media.

Slides were stained for glycogen routinely by the periodic acid-Schiff reagent method; sometimes also with Best's carmine; and, in a few instances, with the iodine vapor method over an I_2-KI solution (*8*).

Other slides were stained for phosphate production by either the alkaline or acid phosphatase technique of Gomori (*9*, *10*), or both. In some instances the incubation mixtures were saturated with phosphate ion as suggested by Maengwyn-Davies *et al.* (*6*). Even at *p*H 6.7, good phosphate production by the alkaline phosphatase technique was observed.

Various controls were employed. Slides were stained prior to incubation as indicated above, and also by Harris' hematoxylin or by hematoxylin and eosin. Heat controls were either 1) placed in hot H_2O at 90–100° C. for 2 to 5 minutes, 2) passed several times through an open flame until thoroughly dried and heated but not charred, 3) treated by both 1) and 2) methods, or 4) kept in a hot oven at 110° C. for 5 minutes. Other slides were freed of glycogen before or after incubation, or both, by saliva or a buffered (citrate-phosphate, *p*H 7.0) malt diastase.

RESULTS

It has been possible to synthesize polysaccharide in sections of frozen tissues, primarily the liver of the rat, sometimes apparently in considerable amount and concentration, as may be seen in figures 1 to 4. Under the following twelve headings, some of the factors and experimental conditions tested for their efficacy in the production of glycogen deposition are given and briefly discussed.

1) *p*H *and buffers.* Success was attained at *p*H 7.4, 6.8 and 6.0. The latter two levels were better than the more alkaline *p*H. The following buffers were employed: Michaelis' veronal buffer (*11*), Cori's 0.3 M glycerophosphate, and 50% saturated acetate [a modification of the technique used by Maengwyn-Davies and Friedenwald (*6*)]. The veronal and acetate buffers gave better results.

2) *Cysteine and glutathione.* Cysteine was used at 0.015 M, as recommended by Cori *et al.* (*5*), or 0.0015 M (*4*), or omitted entirely. Under all three conditions good polysaccharide deposition was attained. Glutathione (2.5×10^{-2} M) seemed to inhibit the reaction in part. Phosphate production was more readily visualized by the alkaline phosphatase technique in the presence of a high cysteine concentration (fig. 5). This result is an additional indication of the origin of the phosphate from phosphorylase activity, in view of the reported inactivation of alkaline phosphatase by cysteine (*12*, *13*).

3) *Na^+ : K^+ ratio.* The effect of Na^+ : K^+ ratio was tested in view of the findings of the Hastings group (*14*, *15*) on the superiority of an intracellular ionic environment for glycogen synthesis in fresh liver slices. When K^+ was substituted for Na^+ in salts of the substrate, no effect favoring polysaccharide synthesis was noted.

4) *Di-valent ions.* Equally good results were obtained with or without the introduction of Ca^{++} at 0.036 M. The presence of Ba^{++} (0.013 M) did not interfere with the reaction, nor did it appreciably improve it. Good results were obtained in the presence of 0.1% $Pb \cdot (NO_3)_2$.

5) *Thickness of section.* Sections cut at 25 to 30 microns gave more consistently positive results than those cut at 15 microns.

6) *Animal variation.* There is some evidence for the influence of individual animal variation. In a few instances, sections from two different animals were placed on the same slide. In some of these cases, sections of one of the livers gave better results than its mate. In successive experimental runs, characteristic patterns of liver glycogen deposition recurred in sections from the same animal.

7) *Cofactors.* Adenylic acid was added because of the findings of the Cori group (*5*), and hexose diphosphate was added as a possible cofactor for phosphoglucomutase activity as described by Maengwyn-Davies *et al.* (*6*). No effects were observed upon addition of these compounds.

8) *Temperature.* No increased deposition was observed when the temperature was lowered from 37° to 30° C.

9) *Fluoride.* The reaction proceeded well and was quite reproducible in the presence of 0.1 M fluoride.

10) *Ethyl alcohol.* Twenty percent ethyl alcohol was tried in an effort to precipitate formed polysaccharide. This did not improve the result, but polysaccharide was formed in this concentration of ethanol. Phosphate patterns were well correlated with polysaccharide deposition.

11) *Glycogen.* It was not necessary to add glycogen to the substrate to obtain good polysaccharide deposition.

12) *Phlorizin.* In the presence of 1×10^{-2} M phlorizin, glycogen deposition was markedly inhibited, and conversely, in the presence of this agent glycogen loss from liver sections (of fed rats) incubated in the absence of glucose-1-phosphate was noticeably retarded.

The pattern in which the polysaccharide was observed in the liver was quite striking. In general, it can be said that the locus of net deposition appeared to be related to the central veins and some of the other blood vessels. Most often, deposition was seen at the periphery of the lobule in the periportal region—roughly in an area equidistant from the central vein (fig. 3). Cases of more extensive deposition, however, did occur (fig. 1).

At times the deposition appeared to be quite distinctly on a cellular basis; that is, individual cells could be readily visualized because of varying amounts in juxtapositional cells. The appearance in these cases resembled in some respects the pattern observed in the liver of some fasting rats. On the other hand, a more diffuse deposition was frequently encountered. In this type, individual cells did not stand out, and one obtained the impression of a wavelike phenomenon in which the center of origin appeared to be an area very low in polysaccharide deposition, *i. e.*, the area immediately surrounding the central vein. In the actual region of deposition, the tissue area showing polysaccharide was sometimes very sharply delimited from the area showing none by a zone of maximum deposition, especially on the centripetal side of the lobule (fig. 3).

The size of the polysaccharide particles were also variable. Sometimes they appeared to be very fine. Not infrequently, however, coarse particles were present that could be detected even at × 125 magnification.

The optimum time of incubation under the experimental conditions employed was from 1 to 6 hours. Overnight incubation (12–20 hours) did not materially increase or decrease net polysaccharide deposits, except in one or two instances.

Sections have been stained by Best's carmine and iodine vapor. The red-brown color given by the latter method in liver sections appears to substantiate the identity of the polysaccharide as glycogen. Furthermore, the deposited material could be digested out by a ten-minute exposure to human saliva.

This method may be applied to other tissues as a histochemical technique for phosphorylase and transglucosidase. In the kidney of the rat, polysaccharide formation has been observed in the proximal convoluted tubules (figs. 6, 7). Abundant polysaccharide was deposited in the uterine muscle of the human being and of rabbit.

When this technique was applied to the liver of fed rats, and to some other tissues normally containing glycogen such as normal cervix and luteal-phase endometrium, the glycogen originally present seemed to disappear in part or *in toto*. In the cervical epithelium, glycogen was deposited in the basal layer or layers where it is not normally visible. When the latter tissues were heated, the glycogen was usually retained in the tissues, giving the impression that in the above instances it was enzymatically removed, rather than dissolved by simple solution. In liver of fed rats there was an intensification of the staining reaction in the periportal areas, just as in the incubated liver of starved rats, but glycogen was observed to diminish or disappear from areas around the central vein in the former. Glycogen can also be deposited in human endometrium, but the physiological state of the individual with respect to the steroid sex hormones may influence the reaction. Further study of this factor is necessary. Endometrial adenocarcinoma and squamous-cell carcinoma of the cervix will in some specimens exhibit glycogen after incubation.

SUMMARY AND CONCLUSIONS

There seems to be no plausible reason to doubt that, in the presence of glucose-1-phosphate and a buffer, sections of frozen liver of starved rats synthesize a polysaccharide, probably glycogen. An apparent net increase of polysaccharide was noted also in the kidney of the rat, in smooth muscle of man and rabbit, in stroma of the endometrium of a rabbit, in glands of the human endometrium, and in the basal layers of human cervix uteri. Presumably this process involves phosphorylase and the branching factor, a transglucosidase (*16*).

Work with crystalline phosphorylase has shown that the reaction must be seeded with glycogen, that cysteine is an activator of the reaction, and that adenylic acid acts as a coenzyme. Potassium ion has been shown

to enhance glycogen-formation more than sodium, in studies with fresh liver slices. However, using frozen liver of starved rats, these conditions are not essential. This is probably only an expression of a closer approximation to the complexity of the normal physiological state of frozen sections than of crystalline enzyme.

Glycogen disappears from some parts of sections of the liver of fed rats, and from human tissues where originally present, under conditions found to favor polysaccharide deposition in liver of starved animals. These findings bring to mind similar work by Hastings and Buchanan (*17*) and Teng *et al.* (*18*), who have also noted a general net decrease of *in vitro* glycogen-formation when the preincubation glycogen levels were high. While the present work is in part a confirmation of this phenomenon, it is pertinent to point out that these effects are very local, though still very definite, and that neighboring cells and cell layers behave in opposite manner. The glycogenolytic reaction, although apparently enzymatic, is not understood and is the subject of further study. Efforts to synthesize glycogen in sections from which pre-existing glycogen had been completely removed by diastase, saliva, or by merely incubating the sections in a buffer, were unsuccessful. The latter phenomena can be interpreted to mean that our preliminary incubation for glycogen digestion resulted in a loss of enzyme and/or some factor or factors essential for its activity.

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PLATE 59

Photomicrographs of sections of frozen liver of the rat. The tissues were cut at 15 to 25 microns and incubated in media containing 0.016 M glucose-1-phosphate as a substrate. × 40

FIGURE 1.—Liver treated with periodic acid-Schiff reagent. Note extensive amounts of deeply staining material.

FIGURE 2.—A similar section incubated in medium lacking only glucose-1-phosphate. Note almost complete absence of polysaccharide.

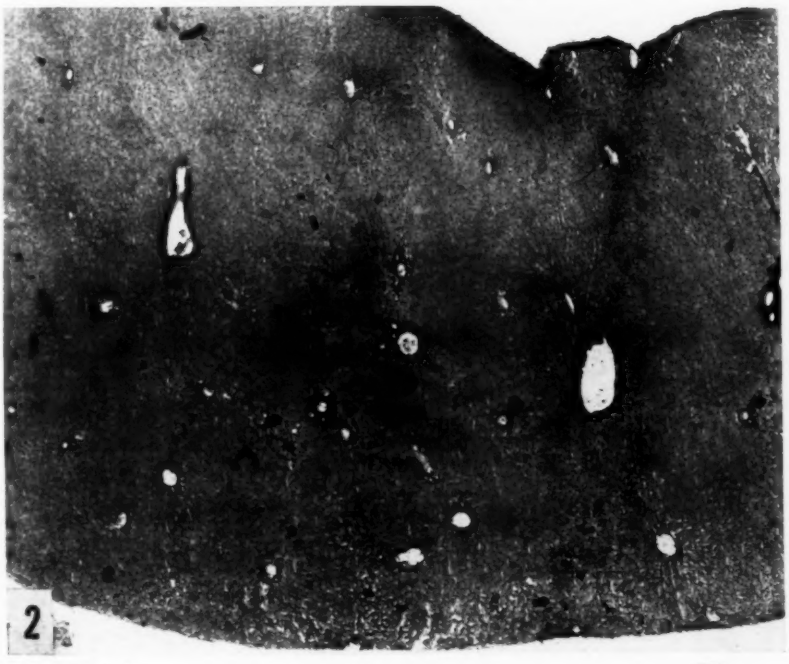

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PLATE 60

Photomicrographs of sections of frozen liver of the rat. Tissues were cut at 15 to 25 microns and incubated in media containing 0.016 M glucose-1-phosphate as a substrate. × 40

FIGURE 3.—Liver treated with periodic acid-Schiff reagent. Note some tendency of colored material to be confined more to the periphery of lobules and greater intensity of reaction at centripetal edge of reacting zone.

FIGURE 4.—Liver stained with Best's carmine. Stained areas, though not so marked, similar to those observed in figure 3.

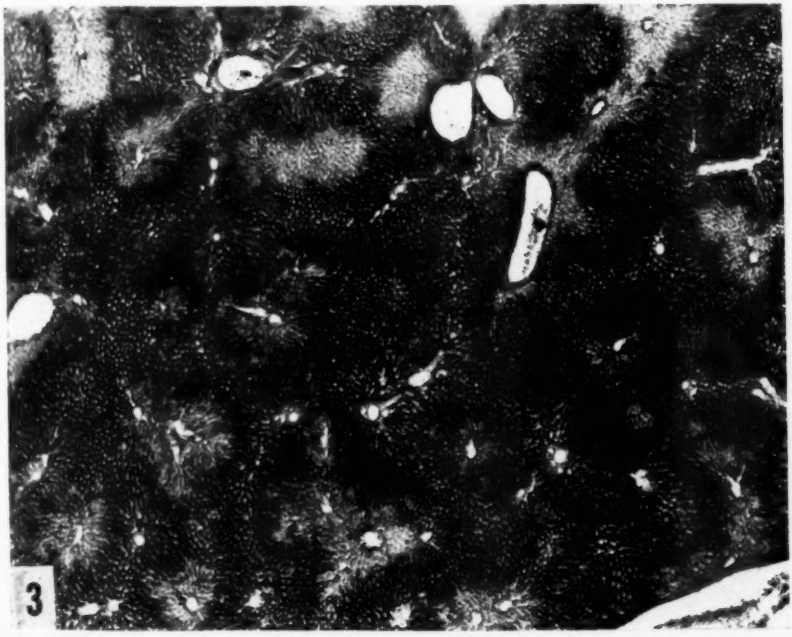
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PLATE 61

Photomicrographs of sections of frozen liver and kidney of the rat. Tissues cut at 15 to 25 microns and incubated in media containing 0.016 M glucose-1-phosphate as a substrate. × 40

FIGURE 5.—Liver treated by a modification of Gomori's phosphatase technique, using cobalt ions as an indicator. Note general similarity of reacting loci to those observed in figure 4.

FIGURE 6.—Kidney stained with Best's carmine. Note stained areas in cortical, extraglomerular regions.

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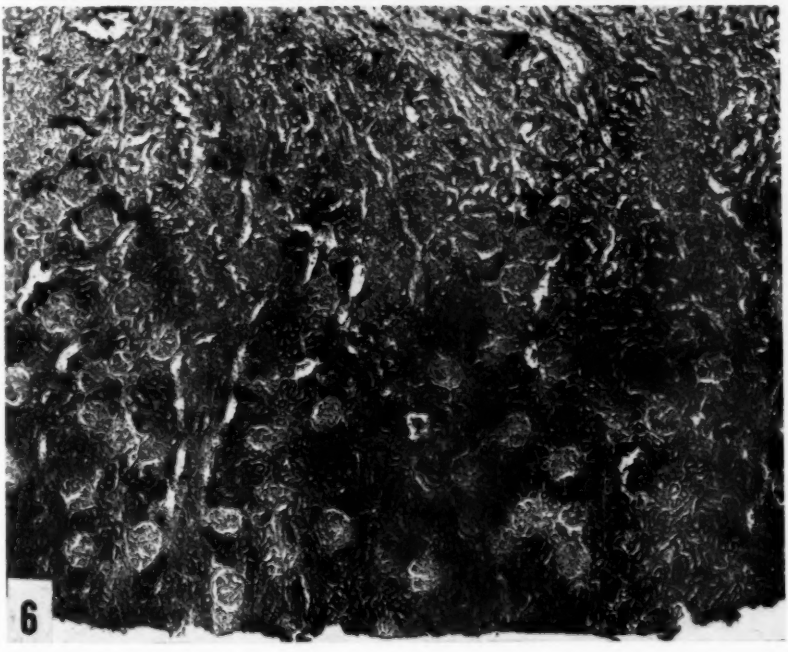
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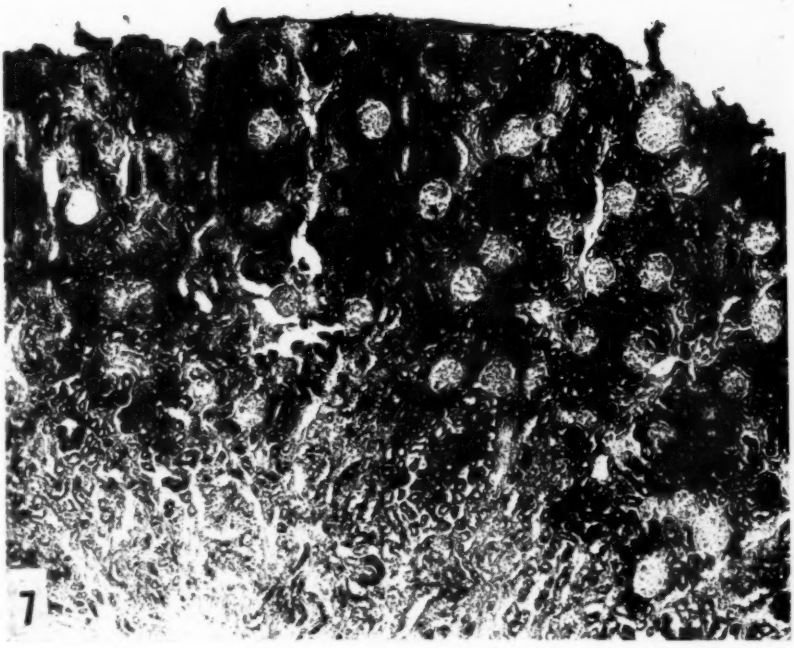
PLATE 62

Photomicrograph of section of frozen kidney of the rat. Tissues cut at 15 to 25 microns and incubated in media containing 0.016 M glucose-1-phosphate as a substrate. × 40

FIGURE 7.—Similar to figure 6. Stained by phosphatase technique. Note correspondence to reacting regions of figure 6.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Biological Studies on Stable and Radioactive Rare Earth Compounds. I. On the Distribution of Lanthanum in the Mammalian Organism [1, 2]

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It has been shown that lanthanum forms complexes with nucleic acids which are insoluble within the physiological *p*H range (*1–3*). This property of lanthanum has been utilized for the fixation of nucleic acids in chromosomes (*4*), for electron staining of chromatin fibrils (*5*), for the analysis (*6*) and the purification (*7*) of nucleic acids. The composition of the complexes formed by lanthanum and other rare earths with desoxyribonucleic acid has been studied and will be reported elsewhere (*8*).

Little is known about the biological effects of the rare earth elements. Björkman and Horsfall (*9*) observed that lanthanum affects influenza virus in a manner similar to that of ultraviolet radiation. It was shown in this laboratory that the growth and metabolism of streptococci is inhibited by lanthanum ions (*10*). The distribution and excretion of several carrier-free, radioactive, rare earth elements, among them lanthanum, was studied in rats by Hamilton *et al.* (*11–13*). The histopathology of carrier-free, radioactive fission product mixtures containing mainly rare earths, and of Ba^{140}–La^{140} mixtures (carrier-free) has been reported (*14*). The half-lethal doses of these irradiating agents have been determined; in the instance of Ba^{140}–La^{140}, the LD_{50} is 4 μc per gram of mouse tissue (*14*).

The object of the present investigation was to study the distribution of lanthanum in normal and tumor-bearing mice as a function of time, dosage, route of administration and chemical state of the element (ionized and nonionized compounds). Stable lanthanum (La^{139}) containing

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[2] This work was performed under Contract AT-30-1-880 between Montefiore Hospital and the United States Atomic Energy Commission.

lanthanum (La^{140}) as a tracer was employed in these studies. A preliminary report on some of these experiments has previously been presented (*15*).

MATERIALS AND METHODS

Biological material.—Albino mice from the Rockland and Carworth colonies and strain A mice (Strong colony) were employed. Animals of both sexes were used, but for individual experiments only mice of the same sex were employed. They were fed Rockland mouse pellets and water *ad libitum*.

The animals were injected with solutions of lanthanum by *intravenous*, *intraperitoneal*, and *subcutaneous* routes. The intravenous injection was given via the tail vein. At selected time intervals the animals were weighed, sacrificed, and the various tissues removed for analysis. In order to improve the statistical significance of the determinations, the respective organs or tissues of 5 or more mice were pooled and then weighed. The organ-pools were minced and 1 gram aliquots were used for the radioassay. Bone samples of femora and humeri were used. In some experiments, after removal of the organ and tissue samples, the remainder of the animal (here designated as carcass) was finely ground and several 1 gram aliquots were assayed for radioactivity. In one experiment, blood for assays was obtained by exsanguination. Histologic and radioautographic studies were performed on small samples of the organs which were fixed in 15 percent formalin-alcohol.

Lanthanum preparations.—Stable lanthanum (La^{139}) was obtained in the form of the chloride ($LaCl_3 \cdot 7H_2O$) of a high degree of purity.[3] The radioactive lanthanum isotope La^{140} was obtained from the Oak Ridge National Laboratory in the form of the oxide (La_2O_3), with stable lanthanum ($La_2^{139}O_3$) as carrier. This material is prepared in the nuclear reactor by neutron bombardment of stable lanthanum oxide. Each irradiated unit consists of approximately 90 mgm. of La_2O_3 with an activity of the order of 40 mc. The half-life of La^{140} is 40 hours, decaying to stable cerium with the emission of β and γ rays of maximum energies of 2.1 mev and 2.3 mev, respectively. The bulk of the lanthanum oxide was transferred from the aluminum container to a glass vessel in dry form, the container was subsequently rinsed with 10 ml. of distilled water and then the lanthanum chloride was prepared by dissolving the oxide by the addition of 10 ml. of 0.2 N HCl to the suspension. This stock solution which contained approximately 3.8 mg. of La per ml., and which was 0.1 N with regard to HCl, served for the preparation of the final solutions employed in the experiments. For the studies on the distribution of lanthanum in ionized form, the stock solution was suitably diluted with physiological saline with or without the addition of further increments of stable lanthanum in the form of $LaCl_3$. In most instances, the *p*H of these solutions was adjusted to a value close to 5 with NaOH. For the

[3] Stable lanthanum chloride and oxide were obtained from Research Chemicals, Inc., Burbank, Calif. In some of the experiments, $LaCl_3$ preparations obtained from the Eimer and Amend Division of the Fisher Scientific Co., New York, were employed.

experiments with lanthanum complexes, the $LaCl_3$ present in the stock solutions was treated with the sodium salt of ethylenediamine tetra-acetic acid, *e.g.* sodium versenate, in the following manner. Solutions of $LaCl_3$ and of sodium versenate[4] containing equimolar amounts of these compounds were mixed in acid solution and the *p*H was adjusted to 7.5–8.0 by means of NaOH in order to insure formation of a stable complex (*16*). The complex was readily soluble under these conditions. Qualitative tests with high polymer sodium desoxyribonucleate (STN) showed that the lanthanum versante fails to react with STN at *p*H values above 7, thus indicating that the lanthanum is present in the form of a tight complex.

The lanthanum content of the stock solutions was checked by a volumetric method involving the precipitation of the lanthanum as the oxalate (*17*).

The tracer dose of radioactivity employed in the majority of the lanthanum-distribution experiments was of the order of 15 to 20 μc.

Assay procedures.—The relatively short half-life of La^{140} necessitates a rapid procedure for the preparation of the samples. Therefore, a wet ashing technique and the use of liquid samples with fixed geometry was adopted. One-gram aliquots of the tissues, or 1 ml. of the body fluids, were placed into small beakers, 3 ml. of concentrated nitric acid were added, and the beakers heated until the tissue was digested. The amount of tissue to be ashed and the volume of acid added were kept constant because of the self-absorption of β particles in the samples which is roughly proportional to the specific gravity of the ash solution. If the weight of the sample was less than one gram, additional tissue, free of radioactivity, was added. After digestion each sample was adjusted to 25 ml. with distilled water, transferred to a Petri dish, and placed beneath a thin glass-walled $\beta\gamma$-G-M tube for counting. These Petri dishes were placed in a reproducible position below the counting tube. The geometrical position being kept constant, the counter sensitivity remains the same for every sample. The high sensitivity to β particles, emitted by this isotope, permits the use of thin-walled G-M tubes.

Measurements were made on a standard 64-scaler connected to a mechanical register and a preset timer. The statistical error of counting was kept below 3 percent. La^{140} standards were prepared by adding a known amount of the radioactive lanthanum solution to 1 gm. of nonradioactive tissue, and by subsequent ashing. The standards were assayed together with the samples. This corrected for decay and afforded a direct measure of relative sample strength.

Radioautography.—The radioautograms were prepared by the contact method, or the emulsion coating procedure. Tissues were fixed in formalin, and paraffin sections were made. In the contact procedure, the

[4] The versenate was obtained in the form of disodiumdihydrogen versenate reagent from the Bersworth Chemical Company, Framingham, Mass. We wish to thank Dr. John J. Singer for placing the material and the information regarding it at our disposal.

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tissue sample was placed in contact with no-screen X-ray film. After exposure, the film was processed in the usual manner.

The coating procedure (*18*) consists of melting a photographic emulsion and painting it over the tissue section mounted on a slide. This insures closer emulsion-section contact and thinner films, giving rise to higher resolutions than the contact procedure. After exposure, *e.g.* for one week, this slide is photographically processed. The resulting radioautogram is superimposed on the tissue section.

OBSERVATIONS AND RESULTS

Administration of Lanthanum in Ionized Form

In these experiments the lanthanum was administered in the form of $LaCl_3$ in solutions of *p*H range 4 to 5, *i.e.*, under conditions in which the lanthanum existed in the form of completely dissociated cations. However, it is possible that upon contact with body fluids, the lanthanum reacts with proteins to form metal complexes in which the rare earth is more tightly bound and possesses a much higher molecular or particle weight than the $LaCl_3$. It was noted that the addition of 1.5 mg. of La as $LaCl_3$ to 1 ml. of serum produced a visible turbidity which is presumably due to the formation of lanthanum proteinates. However, this dose is far in excess of the LD_{50} (*see* Discussion).

Inasmuch as lanthanum is not appreciably absorbed from the gastrointestinal tract (*13*, *19*), only parenteral routes of administration, *viz.*, *intravenous*, *intraperitoneal*, and *subcutaneous*, were employed. Preliminary toxicity tests were carried out prior to the distribution studies. These tests indicated that the half-lethal dose (LD_{50}) of intravenously injected $LaCl_3$ was of the order of 0.01 mg. of La per gm. of body weight of the mouse, while the tolerance toward intraperitoneally administered $LaCl_3$ was considerably greater. $LaCl_3$ doses as high as 0.5 mg. per gram of body weight injected subcutaneously were tolerated.

The *distribution* of $LaCl_3$ injected intravenously into normal mice (Rockland and Carworth) was studied as a function of time and of total La concentration maintaining the radioactivity at tracer levels (15–20 μc).

In order to determine the distribution pattern of the rare earth salt as a function of time, 0.2 ml. of a solution containing 0.06 mg. of La were injected *intravenously* into 18 Rockland mice. The animals were sacrificed in groups of 4 or 5, at intervals from 1 to 144 hours after the injection. The analytical results were computed in terms of the fraction of the total dose of lanthanum injected found in a given organ (percent uptake per organ), and in terms of lanthanum concentration (μg. per gram of tissue). The analysis of the pooled tissues of the first group (see table 1) showed that after 60 minutes approximately half of the administered lanthanum was still in circulation. Twenty hours after the injection the lanthanum uptake by the liver was 74 percent, and then declined to 53 percent after 144 hours. The lanthanum concentration in the "carcass" also showed a decrease, whereas the uptake by bone did not

change appreciably. It is to be noted that although the percentage uptake of the spleen is only about 5 percent, the *concentration* in this organ is comparable to that in the liver, *e.g.* 17 versus 26.4 μg. per gram of tissue. No radiolanthanum could be detected in blood samples obtained 20 hours following the injection. Approximately 5 percent of the total activity is excreted in 40 hours, as judged by a preliminary experiment.*

TABLE 1.—*Distribution pattern of lanthanum after 1, 20, 72, and 144 hours following intravenous administration of 0.06 mg. of La^{+++} as $LaCl_3$*

Time after administration in hours.	1	20	72	144	1	20	72	144
No. of mice	5	5	4	4	5	5	4	4
Tissue	Percent of injected dose in entire organ				Micrograms of La/gm. of tissue			
Liver	.....	74. 3	69. 1	53. 0	.....	40. 98	26. 40	24. 00
Spleen	.....	.....	5. 5	4. 8	.....	.....	16. 98	13. 02
Kidney	.....	0. 6	0. 7	0. 5	.....	1. 44	1. 20	0. 96
Bone*	.....	6. 2	8. 2	6. 0	.....	3. 00	2. 94	2. 76
Carcass†	.....	29. 6	11. 5	11. 7	.....	1. 02	0. 36	0. 36
Blood‡	§49. 7	0	0	0	18. 54	0	0	0

*Bone: Calculated as 6% of body weight.
†Carcass: The remainder of the animals after removal of the tissues listed above.
‡Blood: Calculated as 8% of body weight.
§At 1 hour, the value for blood only is reported. Since the tissues had not been exsanguinated prior to analysis, and since the lanthanum content of blood was found to be high, the values for the tissues were omitted in the table.

Four additional experiments were performed in which amounts of lanthanum chloride corresponding to 0.05 to 0.076 mg. of La were injected into 35 mice. The animals were sacrificed in groups of 5 or more at intervals of 20, 24, and 48 hours, respectively, after injection. These studies yielded essentially similar results, confirming the high lanthanum uptake by liver and spleen.

In order to investigate the distribution of lanthanum in the organism as a function of dose, varying amounts of stable lanthanum (as $LaCl_3$) were added to the radiolanthanum preparations which contained 0.05 mg. of carrier-La per 0.2 ml. of solution. Thus, amounts of lanthanum ranging from 0.05 to 1.25 mg. of uniform radioactivity (15 μc) were injected *intravenously* into 144 Rockland mice. Seventy-two animals were sacrificed 24 hours after injection, while the remainder was allowed to live for 120 hours. Assays of various tissues of the 24-hour groups (see table 2) showed that the percentage uptake of lanthanum per organ remained essentially the same in the liver and muscle in spite of a 25-fold variation in administered dose. The percentage uptake of the kidney, intestine, bone, spleen and lung tissue appeared to vary significantly as a function of the injected dose.

*Note added in proof: Subsequent experiments indicate that approximately 8 percent of the total activity is excreted in the urine and feces within 96 hours following the intravenous injection of 10 μc La^{140}.

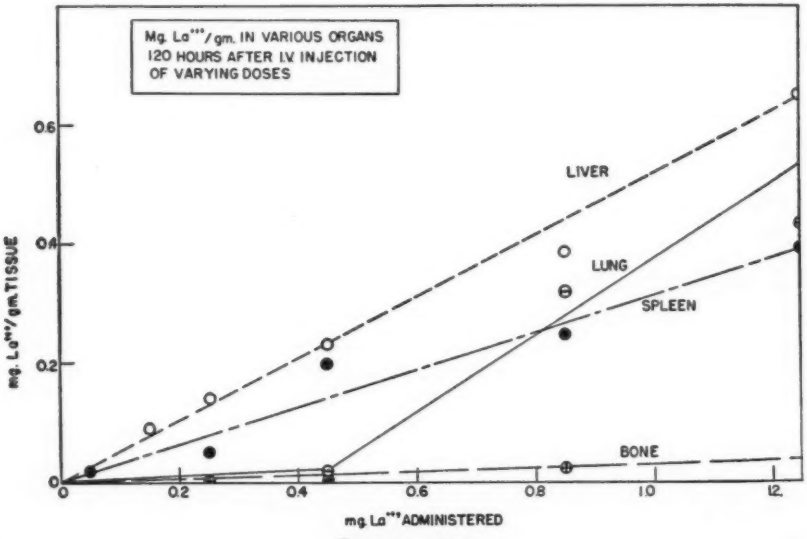
TABLE 2.—*Lanthanum distribution 24 hours after intravenous administration of varying amounts of $LaCl_3$*

Dose of La^{+++} in mg.*	Uptake per organ in percent of dose administered					
	0.05	0.15	0.25	0.45	0.85	1.25
Tissue:						
Liver	69.0	69.0	69.0	81.0	67.0	65.0
Spleen	1.9	2.9	3.5	6.0	5.1	5.3
Kidney	0.8	0.96	0.79	0.37	0.63	0.41
Bone	6.0	4.6	3.0	2.2	2.3	3.6
Muscle	3.0	2.9	3.5	2.2	3.4	3.9
Lung	0.25	0.43	0.44	0.75	8.6	13.0
Intestine	3.7	4.5	5.3	1.5	1.4	1.5

*The activity in all cases was 15 μc.

Since the *percentage* uptake by liver and muscle remained approximately the same, the *absolute* amounts taken up increased proportionately. Thus, the liver may absorb as much as 800 micrograms of lanthanum without an indication that a saturation level has been reached. In contrast to the liver and muscle, tissues such as bone appear to have a more limited capacity for lanthanum uptake. Therefore, the percentage of lanthanum uptake by the skeletal system is highest when the administered dose is lowest (*see* Discussion).

A striking observation made in this experiment is the "*break-through*" of lanthanum into the lung upon increasing the dose administered from 0.45 to 0.85 mg. of La as illustrated in text-figure 1. In this range, which is above the LD_{50}, a ten-fold rise in percent uptake by this tissue takes



TEXT-FIGURE 1

place, with a further rise occurring upon increasing the dose to 1.25 mg. of La. At such high dose levels, the concentration of lanthanum in the lung exceeded that of the liver (*see* Discussion).

Radioautograms of sections of various organs were obtained with the aid of the technique described above. Figure 1 is a photograph of tissue sections, and figure 2 represents the corresponding radioautograms. In this case, the animal was injected intravenously with radiolanthanum and the highest lanthanum concentration was found in the liver and spleen, while the activity in kidney, lung, and lymph nodes was barely discernible.

The distribution of lanthanum chloride, when injected intravenously into mice bearing spontaneous mammary adenocarcinoma and transplanted Sarcoma 180, follows the pattern found in normal mice. The concentration of lanthanum in the tumors was similar to that in muscle. Thus, 20 hours after the administration of 0.076 mg. of La with approximately 10 μc activity, 0.96 percent of the La was found in the tumor.

In order to investigate the affinity of La for tumor tissue in man, 0.49 mg. of La (100 μc activity) in the form of $LaCl_3$ dissolved in 5 percent glucose solution was infused into the right femoral artery of a patient prior to amputation of the right limb. This patient had an osteogenic sarcoma above the medial condyle of the right femur. Body surveys indicated localization of a large amount of radioactivity in the area of the liver of the patient with elimination of only traces in the excreta over a 31-hour period. Assays performed on specimens of bone, tumor, and surrounding soft tissues demonstrated the presence of lanthanum. The radioautograms of the fresh bone tissue suggested the preferential localization of radiolanthanum in the epiphysial line, similar to previous observations on animals with other rare earths of the lanthanum group by Hamilton (*11*) and Bloom (*14*).

The fate of *intraperitoneally* administered $LaCl_3$ was studied in the following manner: 0.38 to 2.0 mg. of La of activities ranging from 75 to 300 μc, dissolved in 0.2 ml. of saline was injected intraperitoneally into 34 animals in groups of 4 or 6. The animals were sacrificed at intervals ranging from 24 to 168 hours after injection and various organs and tissue samples were assayed. The results obtained in one of these groups are shown in table 3. It will be noted that only 0.22 percent of the total lanthanum injected was found outside the abdominal cavity (bone, muscle, lung, and heart tissue) while 68.4 percent of the activity was recovered in the abdominal tissues (liver, spleen, kidney, and gastrointestinal tract). The high activity of the gastrointestinal tract is probably due in part to the deposition of lanthanum on its large surface. This assumption is supported by radioautograms obtained from tissue sections of mice sacrificed 24 hours after intraperitoneal injection of lanthanum chloride. As can be seen from figure 3, the lanthanum is localized at or near the surface of the liver, whereas after an intravenous injection the lanthanum is distributed throughout the liver (fig. 2). It may be concluded that the recovery value for the tissues within the abdominal cavity

would be even higher than that shown in table 3 had the peritoneal linings been included in the analysis. Although the excretion of intraperitoneally injected lanthanum by mice has not yet been determined, the recovery of about 70 percent of this material from the abdominal cavity after 168 hours indicates that excretion takes place very slowly.

TABLE 3.—*Distribution of lanthanum 168 hours after intraperitoneal administration of* $LaCl_3$*†

	Percent of injected dose per organ
Abdominal organs:	
Liver	17. 8
Gastrointestinal tract	46. 9
Spleen	3. 0
Kidney	0. 7
Total	68. 4
Extra-abdominal organs:	
Bone‡	0. 06
Muscle§	0. 10
Lung	0. 04
Heart	0. 02
Total	0. 22

*Dose injected: 2.0 mg. La of 200 μc activity.
†Tissues of 4 mice were each pooled for assay. The values as listed are the average of results from 2 experiments.
‡Bone calculated as 6% of body weight.
§Muscle calculated as 45% of body weight.

Additional distribution data obtained in the course of therapeutic experiments on mice bearing ascites tumor will be presented in a subsequent paper (*20*).

Subcutaneously administered $LaCl_3$ is poorly absorbed. Six days after the subcutaneous injection of 0.06 mg. La (20 μc activity), 54 percent of the activity was recovered from the cutaneous tissues excised near the point of injection, while only 0.5 percent of the activity was found in the liver of the animals.

ADMINISTRATION OF LANTHANUM IN NONIONIZED FORM

The object of these experiments was to study the distribution of lanthanum upon administration in the form of soluble complex salts in which the rare earth is present largely or wholly in the nonionized state. Thus far, the properties of such complexes have been mainly investigated in connection with studies on radioactivity decontamination procedures (*21*). We have studied in a preliminary manner one complex salt of this type, namely, lanthanum versenate. The preparation of this complex has been described above.

In one series of experiments, 0.2 ml. of solutions containing between 0.25 and 0.26 mg. of La^{+++} with 15μc La^{140} in the form of the *chloride* and

the *versenate* were injected *intravenously* into 48 normal mice, divided into groups of 5 animals each. The animals were sacrificed 24, 72, and 120 hours following injection. The results of the assays of various tissues of the 24-hour groups and of the 72-hour versenate group are shown in table 4.

TABLE 4.—*Comparison of the distribution of lanthanum when injected intravenously in the form of the chloride and the versenate**

Organ	Chloride	Versenate	
	(24 hours)	24 hours	72 hours
	Percent of injected dose in entire organ		
Liver	69. 4	21. 3	18. 4
Spleen	3. 5	2. 5	1. 7
Bone	3. 0	7. 1	4. 7
Kidney	0. 8	0. 9	0. 6
Muscle	3. 5	23. 7	18. 1
Lung	0. 44	0. 9	0. 6

*Dose: 0.25 mg. La; 15 μc activity.

The distribution of lanthanum, when administered as versenate complex, differs greatly from that of the chloride. The uptake by the liver is smaller while that by muscle is several times greater. The lanthanum content of the tissues after $LaCl_3$ injection remained unchanged during the experimental period. However, the lanthanum content of the tissues of animals treated with versenate declined with time as illustrated by the 72-hour values in table 4. It was found that within 24 hours, an amount corresponding to 33 percent of the injected dose was excreted (26% in the urine, and 7% in the feces). A preliminary experiment was performed with three strain A mice which had been inoculated two weeks previously with Ehrlich ascites tumor cells. They were injected intravenously with lanthanum versenate (0.1 mg. of La, 20 μc activity). Seventeen percent of the injected lanthanum was found in the liver whereas between 2 to 9 percent was found in the ascites fluid.

In order to study the fate of a lanthanum complex upon *intraperitoneal* administration, 18 mice were injected intraperitoneally with 0.25 mg. of La as lanthanum versenate (approximately 13 μc activity). They were sacrificed in groups of 6 animals, at 24, 72, and 120 hours after injection, The distribution of lanthanum in the various tissues of these animals is shown in table 5. The uptake by the liver was 10 to 15 percent of the total amount administered, which is comparable to the results obtained upon intraperitoneal injection of $LaCl_3$. However, in addition to the surface-coating of the liver observed when $LaCl_3$ was injected intraperitoneally (fig. 3), the lanthanum versenate is actually taken up by this organ (fig. 4).

TABLE 5.—*Distribution of lanthanum following* intraperitoneal injections *of lanthanum versenate**

Organ	La uptake per organ in percent of injected dose		
	24 hours	72 hours	120 hours
Liver	13. 1	15. 1	11. 9
Spleen	3. 0	6. 6	3. 1
Kidney	0. 6	0. 8	0. 9
Bone	3. 1	3. 7	3. 3
Muscle	8. 6	8. 1	6. 1
Lung	0. 7	0. 7	0. 7

*Dose: 0.25 mg. of La containing 13 μc activity.

As may be seen from table 5, the lanthanum uptake by bone and muscle is significantly higher after intraperitoneal administration of the versenate as compared with lanthanum chloride (table 3). Assays of the other tissues (spleen, kidney, lung) likewise indicate a somewhat higher uptake of lanthanum versenate. The material recovered (see table 5) accounts for less than one-third of the activity administered. The remainder had presumably been largely excreted rather than stored in the "carcass," since another experiment performed with lanthanum versenate indicated that over 20 percent of the material was eliminated in the feces and urine within 48 hours after intraperitoneal administration.

In addition, an experiment was performed with strain A mice bearing Ehrlich ascites tumors. The mice had been inoculated 14 days previously with tumor cells and had large ascites at the time of the experiment. Three of these animals were injected intraperitoneally with 0.1 mg. of La containing tracer amounts of La^{140} in the form of the versenate complex. Twenty-four hours later, the tissues were assayed in the usual manner. The liver and muscle tissue had taken up approximately 5 to 8 percent, respectively, while the ascites contained 15 to 22 percent. Additional experiments of this type in which larger amounts of radioactive material were injected will be reported in a following paper (*20*).

DISCUSSION

The complexes formed by lanthanum and other rare earth salts with nucleic acids and nucleoproteins *in vitro* suggested the biological studies which are reported here. Inasmuch as nucleoproteins play a fundamental role in the metabolism and multiplication of normal and malignant cells, the investigation of such complex formation *in vivo* is of biological interest. The degree of complex formation *in vivo* is probably dependent upon the availability of the substance to the chemical receptors within the cells (nucleoproteins), the concentration of such receptors, and their turnover rate during cell metabolism and division. Differences in availability to various tissues were observed upon the administration of lanthanum by various routes. Only minimal amounts were absorbed when lanthanum chloride was given by subcutaneous, intraperitoneal,

and oral routes, which explains the low toxicity observed upon such administration.

On intravenous administration of lanthanum chloride, large amounts of lanthanum were taken up by the liver. In the toxic dose range, gross and histologic liver damage was observed. The radioautogram (fig. 2) illustrates the distribution of lanthanum. Cytochemical studies by C. Leuchtenberger (*22*) indicate an interaction of lanthanum with both ribose- and desoxyribose-nucleoprotein within the cell.

The absolute amount of lanthanum administered determines to a considerable degree the percentage distribution within the animal body. Certain tissues, such as liver, appear to have a considerable capacity to absorb lanthanum, whereas others, such as those of the skeletal system, show a smaller percentage uptake the more lanthanum is administered. Hamilton *et al.* (*11, 13*) found a high relative uptake by bone when carrier-free lanthanum^{140} was given. In the experiments reported here, carrier-containing La^{140} was used and the percent uptake by the skeletal system was lower than with the carrier-free material.[5]

Similar observations on the relationship of dose and uptake have previously been reported for iodine and silver (*11, 23*). This observation indicates that the relative concentration of lanthanum and the radiation levels within various organs of an animal may be regulated to a certain extent by varying the amount of the carrier.

Only minute quantities of lanthanum chloride were found in extra-abdominal organs or in the excreta when $LaCl_3$ was administered intraperitoneally (table 3). The radioautogram of the liver of an animal that received lanthanum intraperitoneally shows the lanthanum deposit on the surface only (fig. 3). The low rate of absorption and toxicity on topical application of $LaCl_3$ may be of advantage whenever local effects are desired. This was utilized in the therapeutic administration of lanthanum by intraperitoneal injection into mice bearing Ehrlich ascites tumor cells (*20*).

The distribution pattern of lanthanum depends also upon the state of the element, *i.e.*, whether it is ionized or nonionized. A nonionized complex such as lanthanum versenate appears to have a more uniform distribution in various tissues of the animal than ionized lanthanum chloride: upon intravenous administration of lanthanum versenate smaller amounts were found in the liver and spleen, whereas considerably more of it was found in muscle as compared with the uptake after lanthanum chloride injections (*cf.* table 4). The greater diffusibility of lanthanum versenate is also demonstrated when it is administered intraperitoneally: whereas lanthanum chloride remains localized under these conditions, lanthanum versenate finds its way into the muscles, skeletal system, etc. (*cf.* table 5) and a large amount is excreted in urine and feces.

Another factor likely to affect the distribution pattern is *particle size* (see p. 566) which is difficult to evaluate at present since the *circulating*

[5] The use of rats by Hamilton and of mice in our experiments constitutes a further difference.

form of lanthanum (*e.g.* serum protein complexes) may differ from the form in which the material is *administered* (*e.g.* $LaCl_3$). The observations on radiocolloids reported by Gofman (*24*) suggest that the "breakthrough" phenomenon here observed (see text-fig. 1) may be related to the size of the lanthanum-containing particles. The binding of lanthanum ions by serum proteins *in vitro* is now under investigation.

The reaction of rare earths other than lanthanum with desoxyribonucleic acid (*8*) makes it probable that their *in vivo* behavior will be similar to or identical with that of lanthanum. This would suggest the use of other rare earth isotopes in place of La^{140} if substances of similar chemical properties but of different half-life and radiation characteristics are desired. A large variety of such isotopes has become available in recent years through the fission of uranium (U^{235}), and plutonium (*25*), as well as through reactions involving neutron capture upon irradiation of stable lanthanum, etc., in nuclear reactors (*26*). Some of these isotopes are available in a carrier-free state which may be of advantage if the toxicity of the stable carrier material interferes with the use of high-level radioactivity for therapeutic purposes. Furthermore, the carrier-free radioisotopes may be of value when their distribution patterns are to be modified by adding suitable amounts of carrier material.

SUMMARY

The distribution of lanthanum in normal and tumor-bearing mice was studied as a function of time, dose, route of administration, and chemical state of the element.

Lanthanum chloride, injected intravenously, is taken up rapidly by various tissues and the concentration was found to be highest in liver and spleen, and low in the skeletal system. The concentration in the lung was very low until a critical dose-level was reached, whereupon it rose sharply.

Minimal transport occurred when lanthanum chloride was injected intraperitoneally or subcutaneously. This suggests its intracavitary or intratumoral use.

Lanthanum complexes are distributed more homogeneously than the ionized forms.

The implications for therapy of tumors with rare earth radioisotopes have been discussed.

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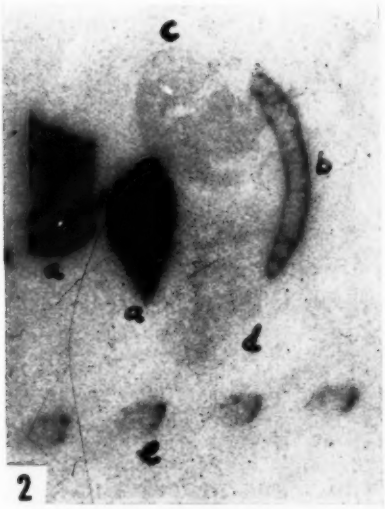
PLATE 63

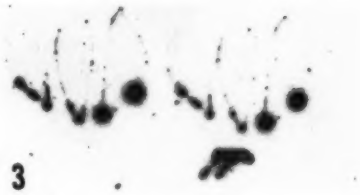
FIGURE 1.—Photomicrograph of organs of a mouse injected intravenously with $LaCl_3$ of 100 μc activity. The sections were taken 5 days after the injection and stained with hematoxylin and eosin. *a*=liver. *b*=spleen. *c*=kidney. *d*=lung. *e*=lymph node.

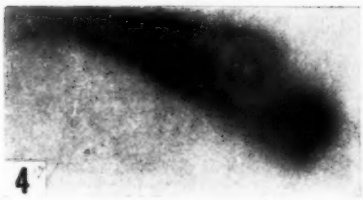
FIGURE 2.—Contact radioautograms of sections shown in figure 1. Note heavy exposure from liver (*a*) and spleen (*b*), and light exposure from kidney (*c*) and lung (*d*). Note that one area of the lymph node shows a heavier exposure.

FIGURE 3.—Contact radioautogram of liver sections of a mouse injected intraperitoneally with radiolanthanum in the form of $LaCl_3$ of 100 μc activity. The animal was sacrified 24 hours after the injection. Note that the radiolanthanum has coated the surface of the liver and that there is no uptake by the liver tissue.

FIGURE 4.—Contact radioautogram of liver section of a mouse injected intraperitoneally with radiolanthanum versenate of 50 μc activity. The animal was sacrificed 24 hours after the injection. In contrast to figure 3 it can be seen that the radiolanthanum was taken up by the liver tissue.







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ANNOUNCEMENT

Fellowships and Grants for Scholars in Cancer Research

Applications for Fellowships in Cancer Research and for Grants for Scholars in Cancer Research are being accepted by the Committee on Growth of the National Research Council, acting for the American Cancer Society.

Fellowships are available for promising postdoctoral students to obtain research training in all branches of biological, chemical and physical sciences and of clinical investigative medicine applicable to the study of growth, typical or malignant. Applications must be received by *December 10, 1952*, to be considered for the year 1953–54. Fellowships ordinarily will begin July 1, 1953, though this date may be varied at the request of the applicant.

Grants for Scholars in Cancer Research are designed to bridge the gap between the completion of fellowship training and the period when the young scientist has thoroughly demonstrated his competence as an independent investigator. A grant of $18,000, payable over 3 years, will be made directly to each Scholar's institution as a contribution toward his support, or his research, or both. These grants are not restricted to the support of individuals who have held American Cancer Society Fellowships. Applications should be submitted by an institution on behalf of a candidate prior to *January 1, 1953*.

Additional information may be obtained from the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue NW., Washington 25, D. C.

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